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13. ABSTRACT (Maximum 200 Words)

Our group has recently characterized a novel autocrine survival pathway in breast carcinoma cells mediated by vascular endothelial growth factor binding to neuropilin-1 and stimulation of the phosphatidylinositol 3-kinase pathway. The goal of this proposal is to define the mechanism by which neuropilin-1 contributes to the survival and metastasis of breast carcinoma cells. Because neuropilin-1 promotes tumor cell survival, we hypothesize that neuropilin-1 plays a critical role in breast cancer metastasis. To investigate this hypothesis, the expression of neuropilin-1 in human breast tumors was examined as a function of disease progression. We found that neuropilin-1 was expressed at a low level in tumor cells but that the level of neuropilin-1 did not increase with disease progression. In addition, our current data suggest that the cytoplasmic domain of neuropilin-1 does not play a role in its pro-survival function. To investigate the ability of neuropilin-1 to directly promote spontaneous metastasis *in vivo*, we have generated a cell line that constitutively downregulates neuropilin-1 expression. Future studies using this cell line will explore the importance of neuropilin-1 expression *in vivo* for the progression of tumorigenic breast carcinoma cells to the metastatic phenotype.

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INTRODUCTION:

The goal of this proposal is to define the mechanism by which neuropilin-1 contributes to the survival and metastasis of breast carcinoma cells. As recently demonstrated by our group, neuropilin-1 enhances the survival of breast carcinoma cells under hypoxic conditions [1]. Hypoxia is one of the most formidable barriers to tumor survival [2]. Although hypoxia will kill most normal cells and some tumor cells, it also provides a strong selective pressure for the survival of the most aggressive and metastatic cells [3-5]. One survival strategy implemented by breast carcinoma cells under hypoxic conditions is the secretion of vascular endothelial growth factor (VEGF) [6-8]. Until recently, the mechanism of VEGF-induced survival was attributed exclusively to its ability to promote angiogenesis by interacting with its classical tyrosine-kinase receptors KDR and Flt-1 on endothelial cells [9-11]. Interestingly, our group has recently characterized a novel autocrine survival pathway in breast carcinoma cells mediated by VEGF [1]. These data are the first to define a VEGF signaling pathway in the survival of breast carcinoma cells by a mechanism independent of angiogenesis. More importantly for this proposal, it was demonstrated that VEGF mediated the survival of these cells by specifically binding to neuropilin-1 and stimulating the phosphatidylinositol 3-kinase pathway [1]. These studies were the first to identify a specific role for neuropilin-1 in tumor cells and suggest that the function and signaling pathways of neuropilin-1 in these cells are distinct from those previously reported in endothelial cells. Based on these findings, we hypothesize that neuropilin-1 plays a critical role in breast cancer metastasis. To investigate this hypothesis, the expression of neuropililn-1 in human breast tumors will be examined as a function of disease progression. The ability of neuropilin-1 to directly promote spontaneous metastasis *in vivo* will also be assessed. In these experiments, the importance of neuropilin-1 expression for the progression of tumorigenic breast carcinoma cells to the metastatic phenotype will be examined. Lastly, the specific signaling events mediated by neuropilin-1 that are responsible for metastasis will be defined. The results of these experiments will likely identify neuropilin-1 as a major participant in the progression of metastatic breast cancer.

BODY:

Specific Aim #1: Investigate the expression of neuropilin as a function of breast cancer progression. *Hypothesis: Neuropilin is preferentially expressed in highly aggressive and metastatic breast tumors.*

Progress Report: Because neuropilin-1 is expressed in highly aggressive and metastatic but not in less aggressive and non-metastatic breast carcinoma cell lines [12], we wanted to investigate the expression of neuropilin-1 in human tumors as a function of breast cancer progression. In collaboration with Dr. Larry Brown, a clinical pathologist at BIDMC, we performed *in situ* hybridization experiments on greater than ten human cases at varying disease stages. We found that neuropilin-1 was expressed at a low level in tumor cells but that the level of neuropilin-1 expression did not increase with disease progression (data not shown). In addition, we found that neuropilin-1 was ubiquitously expressed at a high level in endothelial cells and fibroblasts. Although the expression of neuropilin-1 by endothelial cells was anticipated [12] the presence of neuropilin-1 in fibroblasts was a novel finding. Based on this observation, we hypothesize that neuropilin-1 may play a role in tumor stromal cells to promote metastasis [13]. To

pursue this possibility, we have obtained normal (2VNF) and tumor (1VTF) human breast fibroblast cell lines from Dr. Vimla Band (NEMC, Boston, MA). These cell lines should enable us to investigate in future studies the expression and function of neuropilin-1 in fibroblasts.

In related preliminary experiments, immunohistochemistry for neuropilin-1 protein expression was performed in paraformaldehyde-fixed human breast tissues. However, we were unsuccessful at detecting neuropilin-1 expression with our current neuropilin-1 antibodies. Future studies using frozen tissue sections and /or further optimization of available neuropilin-1 antibodies should overcome this obstacle.

Specific Aim #2: Demonstrate that neuropilin is sufficient and necessary for the progression of tumorigenic breast carcinoma cells to the metastatic phenotype.
Hypothesis: Neuropilin expression is essential for the formation of metastatic tumors in breast carcinoma cells.

Progress Report: To assess the contribution of neuropilin-1 on metastasis *in vivo*, we proposed to assess the ability of ectopically expressed neuropilin-1 in non-metastatic cells that do not express neuropilin-1 and to determine the impact of inhibiting neuropilin-1 function in cells that do express endogenous neuropilin-1. For the overexpression studies, human neuropilin-1 was cloned from MDA-MB-231 breast carcinoma cells with the following primer set: FWD; 5'GATATCCCGCGGATGGATTACAAGGATGACGACGATAAGGAGAGGGGGCT G3' and REV; 5'TCTAGATCATGCCTCCGAATAAGT3' which inserted an amino-terminal FLAG-epitope. FLAG-epitope tagged neuropilin-1 was cloned into $pcDNA_3$ (Invitrogen) as an EcoRV/XbaI fragment and sequenced to determine that it is correct. For retroviral production, FLAG-epitope tagged neuropilin-1 was subcloned into pCLXSN and viruses were generated with the RetroMax System (Imgenex; San Diego, CA). Retroviruses were then utilized to create a stable MDA-MB-453 cell line that constitutively overexpresses neuropilin-1 (APPENDIX, Fig.1). To functionally evaluate the NP-1/MDA-MB-453 cell line, the level of apoptosis was determined. The percentage of AnnexinV-PE^+ cells for the NP-1/MDA-MB-453 cell line was 10% compared to 19% for the GFP/ MDA-MB-453 cell line. This data indicate that the stable overexpression of neuropilin-1 in a cell line that does not endogenously express neuropilin-1 leads to increased cell survival which supports our previous data generated by transient transfections [1]. Following the *in vitro* characterization of this cell line, we initiated experiments to explore their ability to form metastatic tumors in mice. Briefly, the NP-1/MDA-MB-453 cells and control GFP/MDA-MB-453 cells (1×10^6/ 50 µl) were injected into the mammary fat pad of Nude mice (6 mice/group). At 10 weeks following injection, no noticeable signs of tumor formation were present in any of the injected mice and consequently, two mice from each group were sacrificed and their mammary glands were whole-mounted. Histological analysis revealed normal tissue architecture and no indication of tumor formation or tissue disturbance in any of the four mice examined. At this time, the study was ended and the remaining mice were sacrificed by CO_2 inhalation. Although we cannot definitively explain our lack of tumor formation in this study, several possible explanations include injection of too few cells, failure to target the mammary fat pad at the time of injection, and the possibility that the MDA-MB-453

subline used by our laboratory is poorly tumorigenic [14]. Future studies will investigate these possibilities as well as evaluate the rationale behind overexpressing neuropilin-1 in the MDA-MB-435 cell line which expresses a low-level of endogenous neuropilin-1 and displays good tumorigenic and metastatic potential.

Initially, we proposed to explore the impact of inhibiting neuropilin-1 function on metastasis by using a dominant-negative form of neuropilin-1. However, the recent establishment of RNA interference (RNAi) as a technique for reducing the expression of specific genes in mammalian systems [15-17] prompted us to utilize this powerful and highly specific approach. Using sequence-specific small interfering RNA oligonucleotides to neuropilin-1, our laboratory has demonstrated a role for neuropilin-1 in the migration of breast carcinoma cells. Briefly, RNAi abolished neuropilin-1 expression in MDA-MB-435 and MDA-MB-231 cells, and in the presence of a general caspase inhibitor to prevent cell death, it increased their chemotaxis by 1.5 and 2.3-fold, respectively. In addition, reduced neuropilin-1 expression in MCF-7 cells, a poorly migratory breast carcinoma line, enhanced their chemotaxis 5-fold {APPENDIX, Bachelder et al., 2003, Fig. 1C}. These results indicate that neuropilin-1, in addition to its role as a pro-survival gene, acts as an endogenous chemotaxis inhibitor in breast carcinoma cells. Furthermore, we established that the concentration-ratio of vascular endothelial growth factor and Sema3A, competing neuropilin-1 ligands, determines the chemotactic rate of breast carcinoma cells {APPENDIX, Bachelder et al., 2003, Fig. 4}. To continue to explore the significance of neuropilin-1 on metastasis *in vivo*, we have implemented a DNA-vector based strategy for RNAi [18-20] which enables stable and constitutive downregulation of gene expression. Future studies will utilize these cell lines to determine whether neuropilin-1 plays a role in either tumor formation or metastasis of breast carcinoma cells in Nude mice.

The establishment of the RNAi strategy to inhibit gene expression has led to several other laboratory projects and collaborations. Thus far, we have gained considerable insight into the role of the $\alpha 6\beta 4$ integrin in breast carcinoma cells and validated the use of RNAi as a potential therapeutic approach to inhibit carcinoma cell progression. More specifically, two additional publications have resulted from these efforts and are summarized below (Publications Appended):

J. Chung, R.E. Bachelder, **E.A. Lipscomb**, L.M. Shaw, and A.M. Mercurio. Integrin ($\alpha 6\beta 4$) regulation of eIF-4E activity and VEGF translation: A survival mechanism for carcinoma cells. *J. Cell Biol.* 158, 1-11, 2002.
The $\alpha 6\beta 4$ integrin regulates eIF-4E translational activity and VEGF expression leading to a sustained VEGF autocrine-signaling pathway that promotes the survival of breast carcinoma cells.

E.A. Lipscomb, A.S. Dugan, I. Rabinovitz, and A.M. Mercurio. Use of RNA interference to inhibit integrin ($\alpha 6\beta 4$)-mediated invasion and migration of breast carcinoma cells. *(In Press 4/03, Clin. Exp. Metastasis)*
The use of RNAi to inhibit $\alpha 6\beta 4$ integrin expression promotes a decrease in invasion and migration of breast carcinoma cells and suggests that this strategy may be a useful approach to prevent carcinoma cell progression.

Specific Aim #3: Define the downstream signaling events mediated by neuropilin that are responsible for metastasis. *Hypothesis: Neuropilin promotes progression of the metastatic phenotype in breast carcinoma cells by interacting with GIPC, a PDZ domain-containing protein.*

Progress Report: Our group has previously demonstrated that expression of neuropilin-1 in MDA-MB-453 cells promotes survival of these cells in hypoxic conditions. In contrast, MDA-MB-453 cells transfected with a control GFP expression vector were not viable under these conditions [1]. To investigate the involvement of the neuropilin-1 cytoplasmic-domain in this survival, MDA-MB-453 cells were co-transfected with a GFP expression vector and constructs encoding full-length neuropilin-1, truncated neuropilin-1, or a control empty vector. After 15 hours, the transfected cells were serum-starved and maintained in hypoxic conditions (5% CO_2, 3% O_2, 92 % N_2) for an additional 48 hours to induce apoptosis. At this time, the level of cell death in the transfected cells (GFP^+) was analyzed by flow cytometry with AnnexinV-PE staining as described [21]. As previously shown under these conditions, full-length neuropilin-1 decreased the level of AnnexinV-PE^+ staining by 30% compared to cells transfected with a control empty vector. Unfortunately, the level of cell death was comparable for cells transfected with either full-length or truncated neuropilin-1 indicating that the cytoplasmic domain of neuropilin-1 is not required for its survival function. However, this finding does not preclude the possibility that the cytoplasmic domain of neuropilin-1 may be essential for its other functions including the recently identified role of neuropilin-1 as a chemotaxis inhibitor [1]. Future studies will investigate this hypothesis.

The cytoplasmic domain of neuropilin-1 has been shown to interact with GIPC, a PDZ domain-containing protein in olfactory bulb membrane extracts [22]. To address whether this interaction is important in the signaling events mediated by neuropilin-1 in breast carcinoma cells, we attempted to co-immunoprecipitate endogenously expressed neuropilin-1 and GIPC in MDA-MB-231 cells. Using the same lysis buffer as described by Cai and Reed [22], we were unable to co-immunoprecipitate neuropilin-1 and GIPC in two separate experiments (APPENDIX, Fig. 2). Likely explanations for this discrepancy include differences in protein abundance between CNS and carcinoma cell line extracts and in the ability of the GIPC antibody obtained from Dr. Marilyn Gist Farquhar (UCSD), a different antibody than used by Cai and Reed [22], to detect this interaction. Taken together, these data suggest that the cytoplasmic domain of neuropilin-1 does not play a role in its pro-survival function and that a neuropilin-1 and GIPC protein-protein interaction does not endogenously exist in breast carcinoma cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Generated a FLAG-epitope tagged human neuropilin-1 construct
- Implemented a system for retroviral generation and propagation
- Developed a reliable strategy for utilizing RNA interference in transient (oligonucleotide transfections) and stable (retrovirus-mediated) systems
- Using these reagents and techniques, identified neuropilin-1 as a regulator of chemotaxis in breast carcinoma cells {Bachelder et al. 2003, APPENDIX}

- Substantiated the importance of the α6β4 integrin in invasion and migration of breast carcinoma cells using RNA interference {Lipscomb et al. 2003, APPENDIX}
- Demonstrated a direct link between α6β4 expression and 4E-BP1 phosphorylation, VEGF expression, and survival of breast carcinoma cells {Chung et al. 2002, APPENDIX}

REPORTABLE OUTCOMES:

Publications:

R.E. Bachelder, **E.A. Lipscomb**, X. Lin, M.A. Wendt, N.H. Chadborn, B.J. Eickholt, and A.M. Mercurio. Autocrine regulation of carcinoma migration: A novel Semaphorin3A/Neuropilin-1 inhibitory pathway is suppressed by VEGF. *(In Press 6/03, Cancer Res.)*

E.A. Lipscomb, A.S. Dugan, I. Rabinovitz, and A.M. Mercurio. Use of RNA interference to inhibit integrin (α6β4)-mediated invasion and migration of breast carcinoma cells. *(In Press 4/03, Clin. Exp. Metastasis)*

J. Chung, R.E. Bachelder, **E.A. Lipscomb**, L.M. Shaw, and A.M. Mercurio. Integrin (α6β4) regulation of eIF-4E activity and VEGF translation: A survival mechanism for carcinoma cells. *J. Cell Biol.* 158, 1-11, 2002.

Presentations: (November 2002)

"Use of RNA interference to inhibit integrin (α6β4)-mediated invasion and migration of breast carcinoma cells". Division of Cancer Biology and Angiogenesis Data Club, Beth Israel Deaconess Medical Center, Boston, MA 02215.

Retrovirus Repository and Cell Lines:

Retroviruses were generated using the RetroMax System (Imgenex) and stable virus-producing cell lines were generated following viral stock infection and drug-resistant selection.

Retroviral Stocks	Cell Lines Infected/Selected
Neuropilin1-pCLXSN	MDA-MB-453
GFP-pCLXSN	MDA-MB-453; MDA-MB-435
Empty-pCLXSN	MDA-MB-435
β4-pCLXSN	MDA-MB-435
Neuropilin1-pSuper.Retro (RNAi)	MDA-MB-231; SUM 159
SCRNeuropilin1-pSuper.Retro (RNAi)	MDA-MB-231; SUM 159
β4-pSuper.Retro (RNAi)	MDA-MB-231; SUM 159
SCRβ4-pSuper.Retro (RNAi)	MDA-MB-231; SUM 159
Sema3A-pSuper.Retro (RNAi)	MDA-MB-435
SCRSema3A-pSuper.Retro (RNAi)	MDA-MB-435
Empty-pSuper.Retro (RNAi)	MDA-MB-435

CONCLUSIONS:

During the last year, we have gained much insight into the mechanism by which neuropilin-1 contributes to the survival and metastasis of breast carcinoma cells. We have determined that at the mRNA level the expression of neuropilin-1 does not increase in human tumors as a function of breast cancer progression (Aim 1). However, neuropilin-1 expression was detected in fibroblasts associated with these carcinomas and we are very excited to pursue the possible function of neuropilin-1 in these cells with respect to breast cancer progression. To evaluate the importance of the neuropilin-1 cytoplasmic domain in survival and metastasis, we performed survival assays to compare full-length and truncated neuropilin-1. We found that full-length and truncated neuropilin-1 resulted in comparable levels of cell survival when maintained in hypoxic conditions for 48 hours (Aim 3). This finding suggests that the cytoplasmic domain of neuropilin-1 is not necessary for its survival role. Furthermore, we were unable to confirm the interaction of GIPC with the cytoplasmic domain of neuropilin-1 in breast carcinoma cells shown previously in olfactory bulb membrane extracts. The generation of neuropilin-1 overexpressing retroviruses and of sequence-specific RNA oligonucleotides to inhibit neuropilin-1 expression proved to be most valuable. Unexpectantly, a stable MDA-MB-453 cell line that constitutively overexpresses neuropilin-1 did not form tumors when injected into the mammary fat pad of Nude mice (Aim 2). Future studies will examine the tumorigenicity of our MDA-MB-453 subline to potentially explain this finding. Using the RNAi approach, we identified neuropilin-1 as an endogenous chemotaxis inhibitor in breast carcinoma cells. In addition, we found that the ratio of VEGF and Sema3A concentrations determines the chemotactic rate of breast carcinoma cells. Thus, the role of neuropilin-1 in breast cancer progression is extremely complex and is likely to include both a survival and migratory component. Future studies examining these functions *in vivo* should prove invaluable to the mechanistic understanding of neuropilin-1.

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Competing autocrine pathways involving alternative neuropilin 1 ligands regulate chemotaxis of carcinoma cells

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Abbreviations: Ab, antibody, NP1, neuropilin 1, RNAi, RNA interference, SEMA3A, semaphorin 3A, VEGF, vascular endothelial growth factor

Keywords: neuropilin 1, vascular endothelial growth factor, semaphorin 3A, carcinoma migration

Abstract:

Neuropilin 1 (NP1), in conjunction with plexins, promotes axon repulsion by binding to semaphorin 3A (SEMA3A) [1-4]. Although NP1 is expressed in carcinoma cells, its functions have remained elusive, and neither SEMA3A nor plexin expression has been explored in cancer. Here we provide evidence that breast carcinoma cells support an autocrine pathway involving semaphorin 3A, plexin-A1 and NP1 that impedes their ability to chemotax. Reducing SEMA3A or NP1 expression by RNA interference or inhibiting plexin-A1 signaling enhanced migration. Conversely, expression of constitutively active plexin-A1 impaired chemotaxis. The paradox of how breast carcinoma cells expressing these endogenous chemotaxis inhibitors are able to migrate is explained by their expression of vascular endothelial growth factor (VEGF), a NP1 ligand that competes with SEMA3A for receptor binding [5,6]. Finally, we establish that the ratio of endogenous VEGF and SEMA3A concentrations in carcinoma cells determines their chemotactic rate. Our findings lead to the surprising conclusion that opposing autocrine loops involving NP1 regulate the chemotaxis of breast carcinoma cells. Moreover, our data indicate a novel autocrine function for VEGF in chemotaxis.

Introduction:

In addition to the classical VEGF tyrosine kinase receptors, KDR and Flt-1, NP1 serves as high affinity VEGF receptor [7]. NP1 expression on endothelial cells enhances VEGF signaling by increasing the affinity of VEGF for the classical VEGF receptor tyrosine kinase KDR [7]. Interestingly, NP1 expression has also been reported in a variety of tumors in the absence of KDR or Flt-1 (7,8). Based on the established importance of VEGF in tumor progression, our previous studies investigated a role for NP1 in carcinoma cells as a VEGF receptor, in the absence of classical VEGF receptor tyrosine kinases. These studies indicated that NP1 supports a VEGF autocrine signaling pathway that is critical for breast carcinoma cell survival (8).

Of note, NP1 was identified originally in neurons as a receptor for semaphorin 3A, a soluble member of the semaphorin family that plays a critical role in axon guidance [1,2]. The ability of NP1, which lacks consensus signaling domains, to deliver SEMA3A-associated chemorepulsive signals is dependent on NP1 associations with plexins, proteins displaying met homologies [3,4]. While functions for NP1 as a VEGF receptor in tumor cells have been reported [8,9], the possibility that NP1 influences tumor function by supporting signaling through its alternative ligand, SEMA3A, has not been examined. Here, we provide the first evidence for expression of SEMA3A and plexin-A1 in carcinoma cells and demonstrate that these molecules are autocrine inhibitors of breast carcinoma migration. Importantly, we also identify a novel function for VEGF in carcinoma cell migration involving its inhibition of SEMA3A activity.

Results and Discussion:

Given that NP1 is expressed in breast carcinoma cell lines [7,8] (Fig. 1A) and tumors (Fig. 1A), we assessed the potential involvement of this receptor in carcinoma chemotaxis. Surprisingly, a NP1-neutralizing antibody ***increased*** the chemotaxis of MDA-231 cells towards NIH 3T3 conditioned medium two-fold (Fig. 1B). To confirm and extend this finding, we implemented an RNA interference (RNAi) strategy to diminish NP1 expression in each of three breast carcinoma cell lines. Our previous data indicate that NP1 is essential for breast carcinoma survival because it supports VEGF autocrine survival signaling [8]. To evaluate the role of NP1 in migration separately from its requirement for breast carcinoma cell survival [8], NP1 RNAi transfections were performed in the presence of the general caspase inhibitor, ZVAD-FMK. Under these conditions, the inhibition of NP1 expression did not impact cell survival (Fig. 1C). Of note, this RNAi abolished NP1 expression in MDA-435 and MDA-231 cells, and it increased their chemotaxis by 1.5 and 2.3-fold, respectively (Fig. 1C). In addition, this RNAi reduced NP1 expression in MCF-7 cells, a poorly migratory breast carcinoma line, and enhanced their chemotaxis five-fold (Fig. 1C).

SEMA3A inhibits axon outgrowth by binding to NP1 and the NP1 co-receptor, plexin-A1 [3,4]. Based on our finding that NP1 is inhibitory for breast carcinoma migration, we hypothesized that these cells express SEMA3A and plexin-A1. In fact, SEMA3A and plexin-A1 mRNA were detected in each of three breast carcinoma cell lines, as well as in primary breast tumors (Figs. 2A and 3A). We also identified SEMA3A and plexin-A1 protein by immunoblotting proteins extracted from these

samples with a SEMA3A- or plexin A1-specific Ab (Figs. 2A and 3A). To elucidate a function for SEMA3A in breast carcinoma cells, we reduced SEMA3A expression using a SEMA3A RNAi. This RNAi, which reduced SEMA3A expression significantly (Fig. 2B), increased the migration of these cells (Fig. 2B) without influencing cell survival (data not shown). To assess the importance of plexin-A1 in migration, MDA-231 cells were transfected with a plexin-A1 cytoplasmic domain deletion mutant that inhibits SEMA3A signaling [4]. Expression of this mutant in MDA-231 cells enhanced their migration significantly (Fig. 3B). Conversely, expression of a semaphorin homology domain deletion mutant of plexin-A1 that exhibits constitutive activity in neurons [10] inhibited MDA-231 migration (Fig. 3C). None of these reagents influenced cell survival in the presence of ZVAD-FMK (Figs. 3B and 3C). These data indicate that an autocrine pathway involving SEMA3A, NP1 and plexin-A1 impedes the chemotaxis of breast carcinoma cells. Our ability to increase breast carcinoma migration by expressing a dominant negative plexin-A1 suggests that other plexins, if expressed in these cells, cannot support SEMA3A signaling in the absence of plexin-A1 function.

Genes that are inhibitory for cell growth are frequently subject to chromosomal deletion, mutational inactivation or gene silencing in tumor cells [11,12,13]. The ability of breast carcinoma cells to migrate and invade, despite their expression of molecules involved in SEMA3A signaling, suggested that they support a novel mechanism for repressing SEMA3A function. Increased VEGF expression is a hallmark of breast carcinoma progression [14,15]. Until recently, the function of VEGF in tumor progression was thought to relate solely to its angiogenic activity. We were intrigued by the reported ability of recombinant VEGF and recombinant SEMA3A, which exhibit

similar affinities for NP1 and NP1/plexin complexes respectively [3,7], to compete for NP1 binding [5,6]. Based on these findings, we postulated that endogenous VEGF and SEMA3A compete for NP1 binding, and that the ratio of the concentration of these proteins in carcinoma cells is a critical determinant of their chemotactic rate. To determine this ratio, we measured the relative amounts of SEMA3A and VEGF protein in these cells (Fig. 4A). We then compared the ratio of these concentrations to the relative chemotactic rate of these carcinoma cells. As shown in Fig. 4B, MCF-7 cells, which exhibited the lowest chemotactic rate, displayed the highest ratio of SEMA3A to VEGF protein. MDA-435 cells, which were more chemotactic than MCF-7 cells, demonstrated a lower SEMA3A to VEGF concentration ratio (Fig. 4B). The lowest SEMA3A to VEGF ratio was observed in MDA-231 cells, which exhibited the most robust rate of chemotaxis (Fig. 4B).

If the relative concentrations of endogenous VEGF and SEMA3A in breast carcinoma cells determines their chemotactic rate, then altering expression of these NP1 ligands should influence chemotaxis. To reduce VEGF expression, MDA-435 cells were transfected with either a VEGF antisense (AS) or control oligonucleotide. VEGF AS transfection reduced VEGF expression by approximately 50% relative to transfection with the control oligo (Fig. 4C). Importantly, VEGF AS transfection did not reduce SEMA3A expression in these cells (data not shown). Strikingly, the ability of VEGF AS transfectants to migrate toward conditioned medium was reduced relative to that of the control transfectants (Fig. 4C). These transfectants were maintained in the presence of ZVAD-FMK and the reduction in VEGF expression did not influence their survival (Fig. 4C).

If a major role for VEGF in carcinoma cells involves its antagonism of autocrine SEMA3A, then reducing SEMA3A expression in VEGF AS transfectants should offset the decrease in chemotaxis caused by reduced VEGF expression. To decrease SEMA3A expression stably, MDA-435 cells were infected with retroviruses expressing either a SEMA3A-specific or scrambled RNAi (control). Stable infectants that expressed SEMA3A RNAi exhibited a significant decrease in SEMA3A expression relative to cells infected with the control retrovirus (Fig. 4D). These infectants were then transfected transiently with either the VEGF AS or control oligo. Confirming the data in Fig. 4C, VEGF expression in VEGF AS transfectants was reduced by fifty percent. We then determined the ability of these cells to chemotax toward conditioned NIH 3T3 medium. Confirming the data in Fig. 4C, the chemotaxis of cells infected with the control retrovirus was significantly reduced by VEGF AS transfection. Strikingly, the migration of VEGF AS transfectants was restored upon reducing SEMA3A expression with the SEMA3A RNAi-expressing retrovirus. In the presence of ZVAD-FMK, we did not observe an effect of reducing either SEMA3A or VEGF expression on cell survival (data not shown). These data identify VEGF and SEMA3A as antagonistic, autocrine NP1 ligands that regulate breast carcinoma migration.

Materials and Methods:

mRNA detection

mRNA was purified from the indicated cell lines using the RNEasy kit (Qiagen) according to the manufacturer's recommended protocol. RNA (2 μg) was added to RT-PCR reactions containing the indicated primers at a concentration of 0.6 μM. Alternatively, cDNA was generated from carcinoma cells purified from human breast tumors (provided by K. Polyak, DFCI). The conditions for amplifying SEMA3A and NP1 cDNA were as follows: 35 cycles, 95°C, 15 min., 95°C, 30 sec., 55°C, 1 min., 72°C, 1 min., followed by a 72°C, 10 min. final extension step. The conditions for amplifying Plexin-A1 cDNA were as follows: 35 cycles, 95°C, 15 min., 95°C, 30 se., 58°C, 1 min., 72°C, 2.5 min, followed by a 72°C, 10 min. extension step. The sequences of amplification primers are as follows:

SEMA3A Forward: GACTTTGCTATCTTCCGAACTCTTGGGCAC

SEMA3A Reverse: GCTATACATACACACGGCTGATCCCTTG

NP1 Forward: ATGGAGAGGGGGCTGCCG

NP1 Reverse: CTATCGCGCTGTCGGTGTA

Plexin-A1 Forward: GAGGATGCCGACATGTTCGGCTTCG

Plexin-A1 Reverse: AGGGCGTCATGGGCACGCACGG

RNAi transient transfections

RNAis were designed and synthesized by Dharmacon, Inc (see below for sequences). Cells at 60% confluency were transfected in penicillin/streptomycin-free medium with the indicated RNAi using TKO lipid (Mirus), following the manufacturer's recommended protocol. The following RNAi concentrations were determined to be optimal for

inhibiting protein expression: MDA-231- NP1 RNAi, 200 nM, SEMA3A RNAi, 200 nM; MDA-435 and MCF-7- NP1 RNAi, 200 nM, SEMA3A RNAi, 100 nM. After 20 hours, RNAis were removed, and the cells were maintained in complete medium with the indicated antibodies for an additional 24 hours.

NP1 RNAi: GAGAGGUCCUGAAUGUUCCTT

Scrambled NP1 control: AGAGAUGUAGUCGCUCGCUTT

SEMA3A RNAi: AAAGUUCAUUAGUGCCCACCU

Scrambled SEMA3A control: AAGUGCACGCCUCUAUAUAUC

SEMA3A and SCR SEMA3A RNAi retrovirus generation

To create SEMA3A-pSUPER and SCR SEMA3A-pSUPER expression vectors, the following oligonucleotides (Invitrogen; Grand Island, NY) were cloned into pSUPER (a gift from R. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands:

SEMA3A,

5'gatccccAGTTCATTAGTGCCCACCTttcaagagaAGGTGGGCACTAATGAACTttttgg aaa3' and

5'agcttttccaaaaaAGTTCATTAGTGCCCACCTtctcttgaaAGGTGGGCACTAATGAACT ggg3';

SCR SEMA3A,

5'gatccccGTGCACGCCTCTATATATCttcaagagaGATATATAGAGGCGTGCACttttgg aaa3' and

5'agcttttccaaaaaGTGCACGCCTCTATATATCtctcttgaaGATATATAGAGGCGTGCAC ggg3'.

EcoRI- and XhoI- digested inserts containing the H1-RNA promoter and targeting oligonucloetides from pSUPER were then subcloned into pSUPER.retro (Oligoengine; Seattle, WA). All plasmids were sequenced to confirm that they were correct.

To generate retroviruses, SEMA3A- or SCR SEMA3A-pSUPER.retro and expression plasmids containing proteins required for viral propagation (Imgenex; San Diego, CA) were tranfected into 293T cells. Viral supernatants were harvested and MDA-MB-435 recipient cells were infected in the presence of 8 μg/ml polybrene (Sigma; St. Louis, MO). Following infection for 24 hours, resistant cells were selected with puromycin (2 μg/mL).

DNA transfections

Cells were transfected with Lipofectamine (Gibco), ZVAD-FMK, a β-gal-expressing plasmid (1 μg) and either VSV-tagged dominant negative human Plexin-A1 (PlexinΔcyt, provided by P. Comoglio) or myc-tagged constitutively active murine PlexinA1 (1 μg PlexA1ΔSem, provided by S. Strittmatter). The ability of these transfectants to migrate toward conditioned medium was assessed after 48 hours in the presence of ZVAD-FMK.

Chemotaxis assays

Chemotaxis toward conditioned NIH3T3 medium was assessed using collagen (Cohesion; 15 μg/mL)-coated Transwell chambers, as described previously [16].

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Figure Legends:

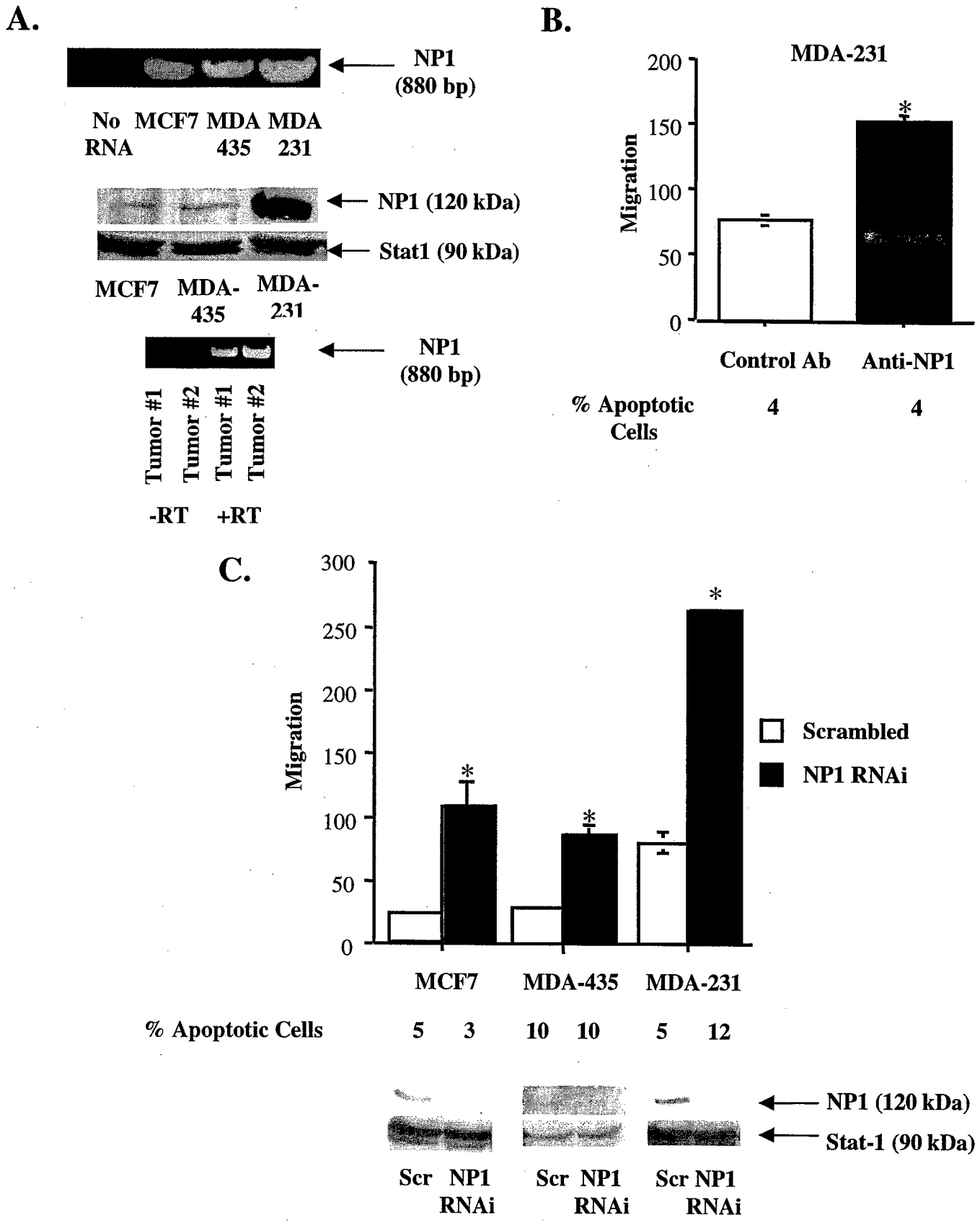
Figure 1: NP1 suppresses chemotaxis of breast carcinoma cells. (A) Top panel: NP1-specific RT-PCR reactions were performed using the indicated mRNAs. Middle panel: Protein extracts were immunoblotted with the indicated antibodies (mouse anti-NP1, Santa Cruz; rabbit anti-Stat1, Santa Cruz). Bottom panel: NP1 was PCR-amplified from cDNA obtained from carcinoma cells isolated from breast tumors. (B) MDA-231 cells were incubated for 6 hrs (+ ZVAD-FMK) with either a rabbit IgG or NP1-specific polyclonal antibody (provided by Alex Kolodkin), and their chemotaxis toward conditioned 3T3 medium was assessed in a 3 hour assay in the continued presence of antibody (+ZVAD-FMK). (C) Cells were transfected with a scrambled or NP1-specific RNAi, and their ability to migrate toward conditioned 3T3 medium was assessed in a 24 hour (MCF7), 15 hour (MDA-435) or 3 hour (MDA-231) assay. The mean number (+/- standard deviation) of migrated cells from two wells (4 fields/well) is indicated. An asterisk (*) indicates a p value < 0.02 in a Student's t-test. Inhibition of NP1 expression by NP1 RNAi was assessed by immunoblotting, as described in (A). The percent apoptotic cells was assessed using Annexin V-FITC, as previously described [17]. Similar results were observed in 3 independent experiments.

Figure 2: Autocrine SEMA3A impedes chemotaxis. (A) Top panel: SEMA3A-specific RT-PCR reactions were performed using the indicated mRNAs. Middle panel: Extracted proteins were immunoblotted with the indicated antibodies (Goat anti-SEMA3A, N-15, Santa Cruz; rabbit anti-Stat1, Santa Cruz). Bottom panel: SEMA3A was PCR-amplified from cDNA obtained from carcinoma cells isolated from breast tumors. (B) Cells were transfected with a scrambled or SEMA3A-specific RNAi and their chemotaxis was determined in a 24 hour (MCF7), 15 hour (MDA-435) or 3 hour (MDA-231) assay. SEMA3A and Stat1 expression were assessed as described for (A). The mean number (+/- standard deviation) of migrated cells from two wells (4 fields/well) is indicated. An asterisk (*) indicates a p value < 0.02 in a Student's t-test. Sema RNAi did not influence the survival of any of these cells (data not shown). Similar results were obtained in 4 separate experiments.

Figure 3: Plexin-A1 signaling inhibits chemotaxis. (A) Top panel: Plexin-A1-specific RT-PCR reactions were performed using the indicated mRNAs. Middle panel: Plexin-A1 and Stat1 proteins were detected by immunoblotting (rat anti-plexin-A1, provided by H. Fujiwasa; rabbit anti-Stat1, Santa Cruz). Bottom panel: Plexin-A1 fragments were amplified from cDNA isolated from carcinoma cells purified from breast tumors. (B) MDA-231 cells were co-transfected with a β-gal-expressing plasmid in addition to either a control plasmid or a VSV-tagged plexin-A1 cytoplasmic domain deletion mutant (plexin-A1ΔCyt), and their migration was measured after 48 hours by X-gal staining. Expression of the VSV-tagged construct was confirmed by immunoblotting with a VSV-specific Ab (Sigma). (C) MDA-231 cells were co-transfected with a β-gal-expressing vector and either a control plasmid or a constitutively active, myc-tagged plexin-A1 construct (plexin-A1ΔSem). The number of migrated cells after 48 hours was assessed by X-gal staining. Expression of the myc-tagged construct was confirmed by

immunoblotting with a myc-specific Ab (Sigma). For B and C, the mean number (+/- standard deviation) of migrated cells from two wells (4 fields/well), as well as the percent apoptotic cells, are indicated. An asterisk (*) indicates a p value < 0.02 in a Student's t-test. Similar results were obtained in 2 trials.

Figure 4: Relative concentration of VEGF and SEMA3A determines chemotaxis rate. (A) SEMA3A expression in equivalent amounts of total cellular protein extracted from MCF7, MDA-435 and MDA-231 cells was assessed by immunoblotting and quantified by densitometry. VEGF expression (pg) in 100 μg of total proteins extracted from these cells was measured by ELISA (R&D). (B) The ratio of relative levels of SEMA3A and VEGF was determined for the indicated breast carcinoma cell lines. In addition, the rates of chemotaxis for these carcinoma lines were assessed by determining the mean number of cells (+/- SD) that had migrated toward conditioned medium in a 16 hour assay. Similar trends were observed in 3 trials. (C) MDA-435 cells were transfected with either a VEGF antisense (AS) or sense (control) oligonucleotide as previously described [8] (+ZVAD-FMK), and their migration toward conditioned medium was assessed in a 15 hour assay. The percentage of apoptotic cells was measured as described for Fig. 1. (D) MDA-435 cells were infected stably with either a SEMA3A RNAi-expressing or control retrovirus. These retroviral cells were then transfected transiently with either a control or VEGF antisense (AS) oligo (+ZVAD-FMK). The ability of these cells to migrate toward conditioned medium was determined in a 15 hour assay. For C and D, the mean number (+/- SD) of migrated cells from two wells (4 fields/well) was determined. An asterisk (*) indicates a p value < 0.02 in a Student's t-test. VEGF and SEMA3A expression were assessed as described for (A). Similar results were obtained in two separate experiments.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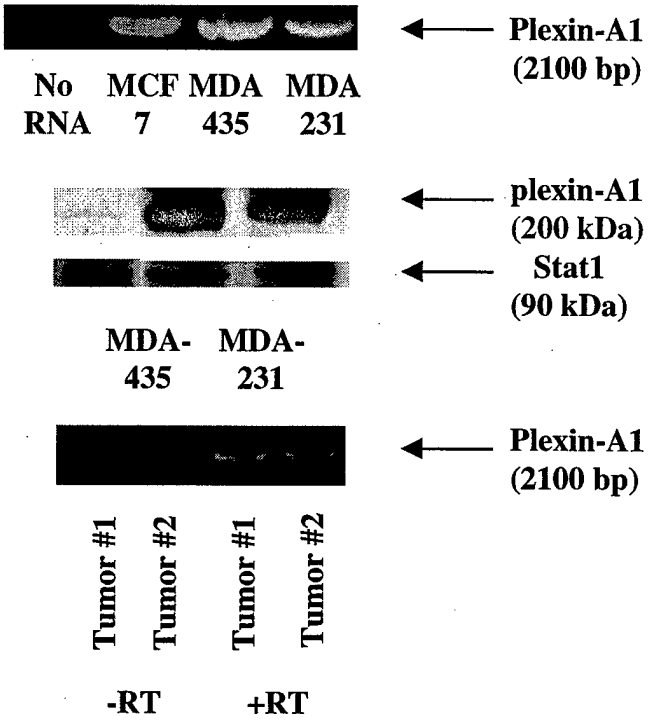
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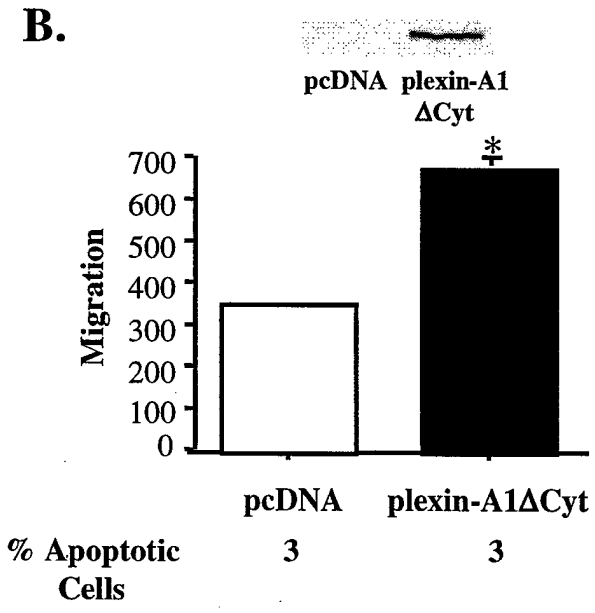
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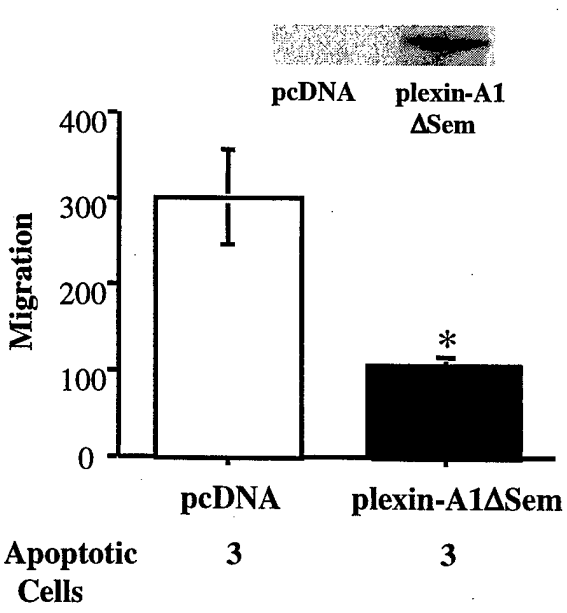
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Use of RNA interference to inhibit integrin ($\alpha6\beta4$)-mediated invasion and migration of breast carcinoma cells

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Key words: $\alpha6\beta4$, adhesion, integrin, invasion, ligand-independent, migration, small interfering RNA, RNA interference

Abstract

The application of small interfering RNA (siRNA) oligonucleotides to silence gene expression has profound implications for the intervention of human diseases including cancer. Using this technique, we explored the possibility that the $\alpha6\beta4$ integrin, a laminin adhesion receptor with a recognized role in the invasive phenotype of many carcinomas, represents a potential therapeutic target to inhibit the migration and invasion of carcinoma cells. We found that siRNA oligonucleotides targeted to either subunit of the $\alpha6\beta4$ integrin reduced cell surface expression of this integrin and resulted in decreased invasion of MDA-MB-231 breast carcinoma cells. Interestingly, reduced $\alpha6\beta4$ expression also promoted decreased migration on non-laminin substrata indicating that this integrin can function in a ligand-independent manner. In addition, the absence of $\beta4$ expression in these cells augmented the formation of $\alpha6\beta1$ heterodimers and increased adhesion to laminin-1. Taken together, these results substantiate the importance of the $\alpha6\beta4$ integrin in invasion and migration that has been demonstrated previously by expression of the $\beta4$ subunit in $\beta4$-deficient cell lines and by function blocking antibodies. Furthermore, these data suggest that the utilization of siRNA oligonucleotides to reduce the expression of the $\alpha6\beta4$ integrin may be a useful approach to prevent carcinoma cell progression.

Abbreviations: BSA – bovine serum albumin; DMEM – Dulbecco's modified Eagle's medium; IRS – insulin receptor substrate; LPA – lysophosphatidic acid; PBS – phosphate-buffered saline; PE – phycoerythrin; PI3-K – phosphatidylinositol 3-kinase; RIPA – radioimmune precipitation buffer; RNAi – RNA interference; siRNA – small interfering RNA; si-$\alpha6$ – siRNA oligonucleotides for $\alpha6$; si-$\beta4$ – siRNA oligonucleotides for $\beta4$; si-Inv – inverted-sequence oligonucleotides for $\beta4$; si-Scr – scrambled-sequence oligonucleotides for $\alpha6$; TBS – tris-buffered saline.

Double-stranded RNA triggers sequence-specific post-transcriptional gene silencing in a wide variety of organisms [1–3]. This naturally occurring process, referred to as RNA interference (RNAi), has recently been established as a powerful technique for reducing the expression of specific genes in mammalian systems. RNAi is induced in mammalian cells by introducing exogenous 21-nucleotide RNA duplexes or small interfering RNA (siRNA) oligonucleotides that are homologous to the desired gene [4–6]. Because RNAi induction offers great promise as a gene-specific therapeutic tool for a host of disease conditions, we investigated whether gene silencing by siRNA oligonucleotides could be a novel approach to inhibit the migration and invasion of carcinoma cells. As an initial step to addressing this question, we utilized siRNA oligonucleotides for the $\alpha6\beta4$ integrin, a receptor that has been implicated in the progression of many carcinomas [7].

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The $\alpha6\beta4$ integrin is expressed primarily on the basal surface of most epithelia, and in a few other cell types [7, 8]. $\alpha6\beta4$ is defined as an adhesion receptor for most of the known laminins [9], although increasing evidence indicates that it can also signal independently of ligand binding [10, 11]. The $\beta4$ extracellular domain associates exclusively with the $\alpha6$ subunit to form $\alpha6\beta4$ complexes [12] whereas the $\alpha6$ subunit also associates with the $\beta1$ subunit to form $\alpha6\beta1$ heterodimers [9]. A primary function of $\alpha6\beta4$ is to maintain the integrity of epithelia through its ability to mediate the formation of stable and rigid structures termed hemidesmosomes on the basal surface that link the intermediate filament cytoskeleton with laminins in the basement membrane [13].

Although the involvement of $\alpha6\beta4$ in hemidesmosome organization and function has dominated the study of this integrin, the $\beta4$ subunit was initially identified as a tumor-related antigen associated with metastasis [14]. More recently, it has been demonstrated that the expression of $\alpha6\beta4$ is maintained or even increased in several types of invasive and metastatic carcinomas and that the $\alpha6\beta4$ expression level actually correlates with the progression of these carci-

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nomas [7]. These correlative data have been substantiated by functional studies that have defined a pivotal role for $\alpha6\beta4$ in migration and invasion of carcinoma cells through its ability to interact with F-actin and activate key signaling pathways [15]. Furthermore, $\alpha6\beta4$ promotes survival in carcinoma cells lacking functional p53 protein [16]. The importance of $\alpha6\beta4$ in survival has recently been extended by the report that $\alpha6\beta4$ stimulates translation of vascular endothelial growth factor and that this pathway is a mechanism of survival in carcinoma cells [17].

The role of $\alpha6\beta4$ in migration and invasion was initially shown by exogenous expression of this integrin in $\beta4$-deficient colon and breast carcinoma cells [18, 19]. In breast carcinoma studies, $\alpha6\beta4$ promoted invasion in a phosphatidylinositol 3-kinase (PI3-K)-dependent manner [19]. Since these initial studies, $\alpha6\beta4$ has been shown to activate PI3-K and stimulate migration and invasion by both growth factor receptor-dependent and -independent signaling mechanisms. For instance, $\alpha6\beta4$ and ErbB-2, an orphan receptor of the epidermal growth factor family, associate in carcinoma cells [20] and this interaction stimulates PI3-K activity and invasion in a fibroblast model system [21]. An association of $\alpha6\beta4$ with the Met tyrosine kinase in carcinoma cells has also been demonstrated and $\alpha6\beta4$ was reported to be necessary for the invasive functions of Met by acting as an adapter protein to recruit PI3-K for enhanced Met signaling [11]. In a growth factor receptor-independent manner, the insulin receptor substrates (IRS-1 and IRS-2) act as signaling intermediates to link activated $\alpha6\beta4$ with PI3-K, a pathway that leads to an increase in carcinoma invasion [22]. In addition to stimulation of PI3-K signaling, the $\alpha6\beta4$ integrin has also been shown to promote migration by activation of the MAPK pathway [23, 24], the Rac and RhoA GTPases [19, 25], and the nuclear factor of activated T-cells family of transcription factors [26].

Although the data summarized above indicate that $\alpha6\beta4$ plays a pivotal function in the aggressive behavior of carcinoma cells, it is worth noting that this role was deduced from either expression of the $\beta4$ subunit into $\beta4$-negative cells or the use of antibodies to inhibit $\alpha6\beta4$ function. In the current study, we used the more definitive approach of RNAi to target the $\alpha6$ and $\beta4$ subunits of the $\alpha6\beta4$ integrin in breast carcinoma cells. Using this highly specific and efficient approach, we observed a significant reduction in the surface expression of this integrin that correlated with an inhibition of migration and invasion. Interestingly, the impact of reduced $\alpha6\beta4$ expression was apparent on non-laminin substrates, a finding that substantiates the ligand-independent function of $\alpha6\beta4$. These findings indicate that inhibition of integrin expression by siRNA oligonucleotides may be an effective approach to assess integrin function in carcinoma cells, as well as to impede tumor progression *in vivo*.

Materials and methods

Cells

The MDA-MB-231 human breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository at Georgetown University and maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Rockville, Maryland) supplemented with 10% fetal bovine serum (Sigma, St. Louis, Missouri), 100 U/ml penicillin, and 100 μg/ml streptomycin (each from Life Technologies).

siRNA transfections

Oligonucleotide sequences for $\beta4$ integrin were designated as si-$\beta4$ (GAGCUGCACGGAGUGUGUC) and as si-Inv (CUGUGUGAGGCACGUCGAG), an inverted control. $\alpha6$ integrin oligonucleotides included si-$\alpha6$ (GGUCGUGACAUGUGCUCAC) and a scrambled-sequence control, si-Scr (AUGCAGAGUGGCGCUCUCU). Oligonucleotides were synthesized by Dharmacon Research, Inc. (Lafayette, Colarodo). Cells ($0.5–2.0 \times 10^5$) were plated onto 35-mm tissue culture dishes 24 h prior to transfection with 200 nM of siRNA duplex using 25 μg of TransIT-TKO transfection reagent (Mirus, Madison, Wisconsin) in the presence of serum as described by the manufacturer. One day following transfection, the transfection medium was aspirated from the cells and replaced with fresh complete growth medium and incubated for an additional 48–72 h. For each transfection, either immunoblotting or flow cytometry was used to confirm reduced protein expression of the targeted gene.

Cell Surface Biotinylation and Immunoprecipitation

Following transfection for 3–4 days, MDA-MB-231 cells were washed two times each with phosphate-buffered saline (PBS) and HEPES buffer (20 mM HEPES, 130 mM NaCl, 5 mM KCl, 0.8 mM Mg Cl_2, 1.0 mM $CaCl_2$, pH 7.45). The cells were then incubated on ice with HEPES buffer containing EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, Illinois) at 0.5 mg/ml for 30 min. Each dish was washed three times with HEPES buffer and the cells were lysed in ice-cold radioimmune precipitation (RIPA) buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% Nonidet-P40, 1% deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, leupeptin, and pepstatin) for 15 min at 4 °C. Cell lysates were clarified by centrifugation at $10,000 \times g$ for 10 min, the supernatants collected, and the total protein concentration of each lysate determined by the Bio-Rad DC protein assay (Hercules, California).

Immunoprecipitations following cell surface labeling were performed with equal amounts of total protein or 60 μg for $\alpha3$ and $\beta1$ integrin reactions and 125–150 μg for $\alpha6$ and $\beta4$ integrin samples. Lysates were preabsorbed with either rat IgG whole molecule agarose (Sigma) or, for mouse antibodies, protein G-Sepharose (Amersham, Piscataway, New Jersey) and then incubated with 1 μg of an anti-integrin

antibody overnight at 4 °C. The following antibodies were used for immunoprecipitation: 439-9B, rat anti-β4 integrin mAb (obtained from Rita Falcioni, Regina Elena Cancer Institute, Rome, Italy); GoH3, rat anti-α6 integrin mAb (Immunotech, Westbrook, Maine); MC13, mouse anti-β1 integrin mAb (obtained from Steve Akiyama, NIH, Research Triangle Park, North Carolina); P1B5, mouse anti-α3 integrin mAb (Life Technologies); as well as rat and mouse IgG (Sigma). Immune complexes were precipitated with rat IgG agarose or protein G-Sepharose, washed four times with RIPA buffer, and eluted in 1X reducing sample buffer (biotinylated α6 and β4 integrin immunoprecipitations) or 1X non-reducing sample buffer (biotinylated α3 and β1 integrin immunoprecipitations).

Immunoblotting

For preparing whole cell lysates, cells were rinsed twice with PBS and lysed in RIPA buffer as described above. Lysates and immune complexes were separated by SDS-PAGE and transferred to Hybond-C nitrocelluose membranes (Amersham). Membranes were incubated in block buffer consisting of Tris-buffered saline (TBS) containing 5% nonfat dry milk followed by antibody incubation in TBS containing 1% nonfat dry milk and 0.05% Tween-20. The blots were incubated with a 1:5000 dilution of rabbit polyclonal anti-β4-integrin (505) [15] or 0.5 μg/ml anti-β-actin (Sigma) followed by 0.04 μg/ml peroxidase-conjugated donkey anti-rabbit secondary antibody (Jackson Immnoresearch, West Grove, Pennsylvania). For biotinylation studies, the membranes were incubated in block buffer overnight and then labeled with 0.2 μg/ml peroxidase-conjugated streptavadin in block buffer containing 0.05% Tween-20 for 2 hours at 25 °C. All membranes were visualized by chemiluminescence (SuperSignal West Pico, Pierce). Densitometry was performed using IP Lab Spectrum (Webster, New York) computer software.

Apoptosis assay

Four days following transfection, cells were collected and their level of apoptosis was assessed using AnnexinV-phycoerythrin (PE) (Pharmingen, San Diego, California). Briefly, cells were washed one time each in 1X PBS and IX annexin buffer (10 mM Hepes-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and then incubated for 15 minutes at room temperature with 5 μg/ml AnnexinV-PE. Following incubation, cells were washed once with 1X annexin buffer and analyzed by flow cytometry.

Migration and invasion assays

For chemotaxis assays, the upper and lower surface of the membrane in each Transwell chamber (Costar, Cambridge, Massachusetts) were coated overnight at 4 °C with 15 μg/ml of collagen I (Vitrogen, Palo Alto, California). To prepare the Transwell membranes for invasion assays, 0.5 μg of Matrigel (Collaborative Research, Bedford, Massachusetts) was diluted with cold water and dried onto each filter overnight at 25 °C. For both chemotaxis and invasion assays, Transwell membranes were blocked on the following day with DMEM for 1 hour at 37 °C. Cells were trypsinized and resuspended in DMEM containing 0.25% heat-inactivated lipid free bovine serum albumin and a total of 1×10^5 cells was added to the upper chamber of each well. NIH3T3 conditioned medium or 100 nM lysophosphatidic acid (LPA) (Sigma) was added to the lower chamber of each well. After incubating for 1 hour (chemotaxis assays) or 4 hours (invasion assays) at 37 °C, nonmigrating cells were removed from the upper chamber with a cotton swab. Cells that had migrated to the lower surface of the membrane were fixed with 100% methanol and stained with 0.2% crystal violet in 2% ethanol. Migration was quantified by counting cells per square millimeter using bright-field optics.

Adhesion assays

For adhesion assays, 96-well tissue culture plates were coated overnight at 4 °C with 20 μg/ml laminin-1 (prepared from the EHS sarcoma provided by Dr Hynda Kleinman, NIDR, Bethesda, Maryland) or 1% BSA as a negative control. The wells were then washed 3 times with PBS and blocked for 10 min at 37 °C with 250 μg/ml heat-inactivated lipid free BSA in DMEM. Cells (3×10^4) were resuspended in DMEM and added to the protein-coated wells. After a 1 hour incubation at 37 °C, the wells were washed 3 times with PBS, fixed for 10 min with methanol, stained with a solution of 0.2% crystal violet in 2% ethanol, and washed 3 times with water. The crystal violet stain was solubilized with a 1% SDS solution and the adhesion was quantified by measuring the absorbance at 595 nm. To control for nonspecific adhesion, the mean absorbance (four wells per transfection condition) of the BSA-coated wells was subtracted from the mean absorbance of the laminin-coated wells in each experiment. The BSA-corrected adhesion obtained in the cells transfected with sequence-inverted β4 siRNA oligonucleotides was designated as equal to one and used to determine the fold induction of untransfected and β4 siRNA oligonucleotide transfected cells.

Results

siRNA oligonucleotides for β4 (si-β4) decrease $\alpha6\beta4$ cell surface expression

We used MDA-MB-231 cells to assess the potential usefulness of RNAi as an approach to inhibit $\alpha6\beta4$ function in carcinoma cells. These invasive and metastatic breast carcinoma cells express relatively high levels of $\alpha6\beta4$, as well as a small population of $\alpha6\beta1$ (L. Shaw, personal communication). The total level of endogenous β4 subunit was reduced by approximately 75% 4 days following transfection with si-β4 oligonucleotides compared to control inverted-sequence oligonucleotides (si-Inv) or untransfected (Unt) cells (Figure 1A). Reduced β4 expression was not detected earlier than 96 h (data not shown) indicating that siRNA

oligonucleotides directed at $\beta4$, and possibly other membrane spanning receptors, require several days to suppress protein expression effectively. These data demonstrate that si-$\beta4$ oligonucleotides reduce endogenous $\beta4$ expression in a breast carcinoma cell line.

To determine whether expression of the $\alpha6\beta4$ integrin was suppressed on the cell surface of MDA-MB-231 cells following transfection with si-$\beta4$ oligonucleotides, cells were biotinylated and immunoprecipitated for $\beta4$. $\beta4$ expression was decreased on the cell surface of si-$\beta4$ transfected cells by 70% compared to si-Inv cells and the amount of $\alpha6$ that co-immunoprecipitated with $\beta4$ in these cells was decreased by 66% (Figure 1B). These findings indicate that cell surface expression of $\alpha6\beta4$ is diminished in si-$\beta4$ transfected breast carcinoma cells.

We next investigated whether the surface expression of the $\alpha6$ subunit was altered in si-$\beta4$ transfected cells. Immunoprecipitation of biotin-labeled lysates revealed no significant difference in $\alpha6$ expression between si-$\beta4$ and si-Inv transfected cells (Figure 1C). Moreover, the surface expression of the $\alpha3$ and $\beta1$ integrin subunits was maintained in cells transfected with si-$\beta4$ oligonucleotides (Figure 1C). Isotype-control immunoprecipitations revealed the specificity of the immunoprecipitated protein for each integrin antibody (data not shown). Additional experiments using flow cytometry to identify cell surface integrin expression confirmed these results (data not shown). These data demonstrate that si-$\beta4$ oligonucleotides reduce the expression of $\alpha6\beta4$ on the cell surface of breast carcinoma cells.

si-$\beta4$ oligonucleotides decrease invasion and ligand-independent migration of MDA-MB-231 breast carcinoma cells

To address the functional consequence of reduced $\alpha6\beta4$ expression, we evaluated the ability of MDA-MB-231 cells to invade through Matrigel-coated Transwells following transfection with si-$\beta4$ oligonucleotides. The invasion of si-$\beta4$ transfected cells towards fibroblast conditioned medium was inhibited by 27% compared to cells transfected with the inverted control (Figure 2A). Based on reports that the $\alpha6\beta4$ integrin can impact the survival of carcinoma cells in stress conditions [16, 17], it was important to evaluate the effect of these oligonucleotides on apoptosis. The level of apoptosis was 5% for both si-$\beta4$ and si-Inv transfected cells as determined by the percentage of AnnexinV-PE$^+$ cells (data not shown). This data indicate that reduction of $\alpha6\beta4$ expression does not result in increased apoptosis under these conditions (10% serum) and that the decrease in invasion of si-$\beta4$ transfected cells cannot be attributed to increased levels of cell death.

The ability of $\alpha6\beta4$ to influence the functions of carcinoma cells can occur independently of ligand (laminin) binding [10, 11]. In particular, $\alpha6\beta4$gtimulates the chemotactic migration of carcinoma cells on collagen I but appears to have little impact on their haptotactic migration towards collagen, a process that involves only collagen adhesion receptors [10]. For this reason, we assessed the ability

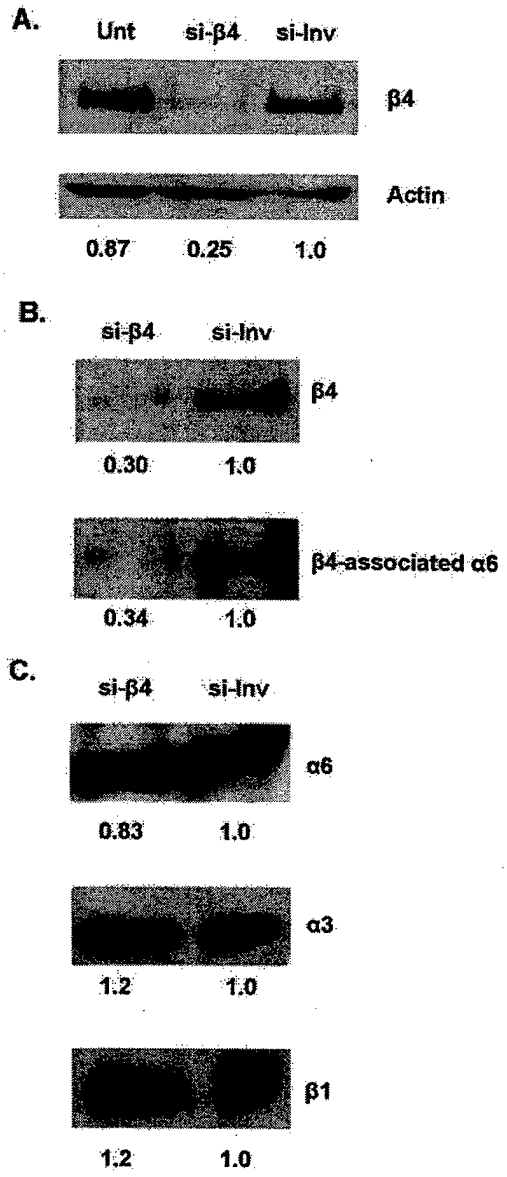

Figure 1. $\alpha6\beta4$ expression is reduced by si-$\beta4$ oligonucleotides. (A) MDA-MB-231 breast carcinoma cells were either untransfected (Unt) or transfected with $\beta4$ siRNA oligonucleotides (si-$\beta4$) or sequence-inverted siRNA oligonucleotides (si-Inv). RIPA extracts were obtained 96 hours post-transfection and equal amounts (30 μg) of total protein were resolved under reducing conditions by 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for $\beta4$ and β-actin. The fold change in $\beta4$ expression was determined by normalizing each band to β-actin and designating the si-Inv lane as equal to 1. Similar results (25–75% reduction in $\beta4$ expression) were observed in greater than 5 independent experiments. Four days following transfection with si-$\beta4$ or si-Inv oligonucleotides, MDA-MB-231 cells were biotinylated, extracted in RIPA buffer, and immunoprecipitated for $\beta4$ (B) or $\alpha6$, $\alpha3$, and $\beta1$ (C), and transferred to nitrocellulose. Immunoprecipitations for $\alpha6$ and $\beta4$ (150 μg of total protein) were separated by 6% SDS-PAGE under reducing conditions whereas $\alpha3$ and $\beta1$ immunoprecipitations (60 μg of total protein) were eluted in non-reducing sample buffer. The membranes were incubated with peroxidase-conjugated streptavadin. The fold change in integrin expression following si-$\beta4$ transfection, when the si-Inv lane is set equal to 1, is indicated. Shown are representative blots from 3 separate experiments.

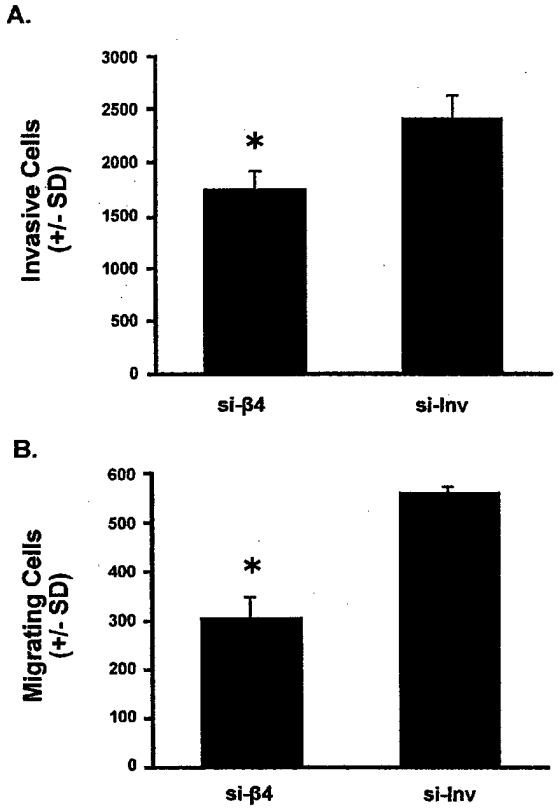

Figure 2. si-$\beta 4$ oligonucleotides decrease invasion and migration in MDA-MB-231 cells. (A) Four days following transfection, the ability of si-$\beta 4$ and si-Inv transfected MDA-MB-231 cells to invade Matrigel towards conditioned fibroblast medium was investigated in a 4 h assay. The results represent the mean number of invasive cells (+/− SD) from two wells (five fields per well). The number of invasive cells was significantly less for si-$\beta 4$ transfected cells than for si-Inv transfected cells (* two-tailed *t*-test, $p = 0.03$). Similar data were obtained in four separate experiments. (B) At 96 hours post-transfection, MDA-MB-231 cells were allowed to migrate through collagen I-coated Transwell membranes for 1 hour towards LPA (100 nM). The data represent the mean number of migrating cells (+/− SD) from 2 wells (5 fields per well). The number of migrating cells was significantly less for si-$\beta 4$ transfected cells than for si-Inv transfected cells (* two-tailed *t*-test, $p = 0.004$). Similar results were obtained in three independent trials.

of MDA-MB-231 cells transfected with si-$\beta 4$ and si-Inv oligonucleotides to migrate towards LPA, a known chemoattractant of breast carcinoma cells [10], on collagen I. The ligand-independent migration of MDA-MB-231 cells was inhibited by 45% when transfected with si-$\beta 4$ oligonucleotides compared to si-Inv oligonucleotides (Figure 2B). Taken together, these observations confirm the importance of $\alpha 6\beta 4$ expression in the migration and invasion of carcinoma cells as demonstrated previously by antibody inhibition experiments and by exogenous expression of this integrin in $\beta 4$-deficient carcinoma cell lines [18, 19].

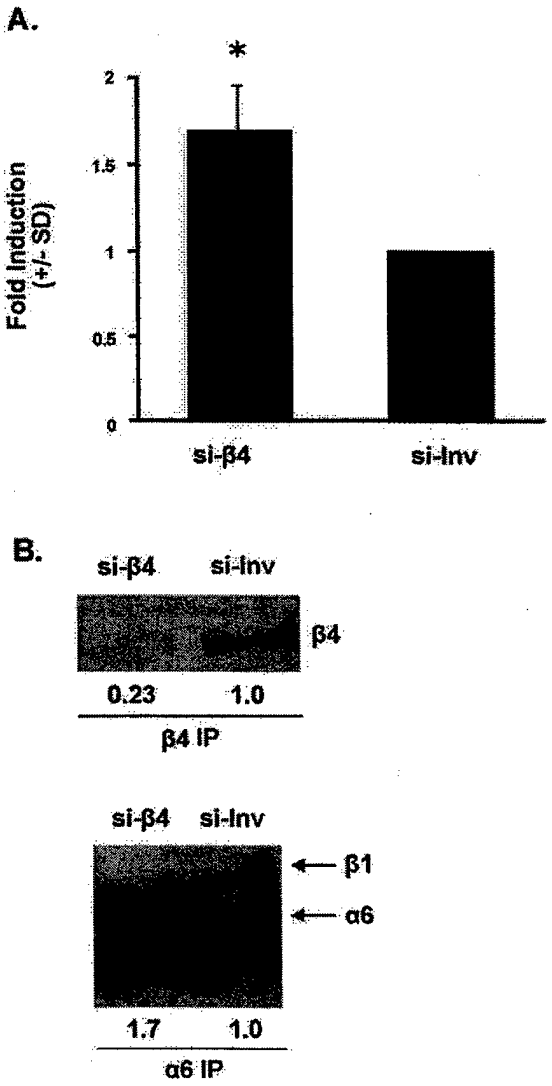

Figure 3. Reduced $\beta 4$ expression promotes adhesion to laminin-1 by enhancing $\alpha 6\beta 1$ formation. (A) MDA-MB-231 cells transfected with si-$\beta 4$ or si-Inv oligonucleotides were incubated for 1 hour in laminin-1 coated wells. Data are the mean fold induction (+/− SD) from three independent experiments, corrected for nonspecific adhesion to BSA, where the adhesion of si-Inv transfected cells was designated as equal to 1. The fold induction of si-$\beta 4$ transfected cells was significantly greater than observed for si-Inv transfected cells (*, two-tailed *t*-test, $p = 0.04$). (B) si-$\beta 4$ and si-Inv transfected MDA-MB-231 cells were biotinylated and lysed 96 h post-transfection and 150 μg of total protein was immunoprecipitated for either the $\beta 4$ (top blot) or $\alpha 6$ (bottom blot) subunit. Immune complexes were separated by 6% SDS-PAGE under reducing conditions and transferred to nitrocellulose. The membranes were then incubated with peroxidase-conjugated streptavadin and visualized by chemiluminescence. The level of $\beta 4$ (top) and $\alpha 6$-associated $\beta 1$ (bottom) expression following immunoprecipitation with the indicated antibodies is shown below each lane where the amount of $\beta 4$ (top) and $\beta 1$ (bottom) in the si-Inv sample is set equal to 1.

Loss of β4 expression leads to increased adhesion to laminin-1 in MDA-MB-231 breast carcinoma cells

Because α6β4 can function as a receptor for laminin-1 in some cells [9], we hypothesized that reduced α6β4 expression by siRNA oligonucleotide transfection would disrupt adhesion to this laminin. However, the level of adhesion to laminin-1 in si-β4 transfected cells was significantly higher (1.7 fold) than observed in si-Inv transfected cells (Figure 3A). Considering that the expression of the α6 subunit remains unchanged in si-β4 transfected cells (Figure 1C and Figure 3B), we reasoned that the reduction of α6β4 might lead to redistribution of the α6 subunit towards α6β1, also a laminin-1 receptor [9]. We found that cells with decreased β4 expression (Figure 3B) had almost twice the level of α6-associated β1 as compared to si-Inv transfected cells as shown by immunoprecipitation of the α6 subunit in the same biotinylated lysates (Figure 3B). Co-immunoprecipitation of the α6 subunit in si-β4 and si-Inv transfected cells followed by immunoblotting for the β1 subunit yielded similar results (data not shown). These findings indicate that in the absence of sufficient levels of β4, α6 forms additional heterodimers with β1 in breast carcinoma cells. These findings indicate that in the absence of sufficient levels of β4, α6 forms additional heterodimers with β1 in breast carcinoma cells.

α6 siRNA oligonucleotides (si-α6) inhibits invasion and migration by reducing α6β4 cell surface expression

MDA-MB-231 cells were transfected with si-α6 oligonucleotides and α6 scrambled-sequence oligonucleotides (si-Scr) to determine whether reduced α6 expression alters β4 cell surface levels and consequently decreases invasion and migration. Three days following transfection, cell surface receptors were biotinylated and cell lysates were immunoprecipitated for α6, β4, and β1. The si-α6 oligonucleotides decreased the expression of α6 by 61% compared to cells transfected with control oligonucleotides (Figure 4A). Interestingly, β4 expression was decreased by 59% in si-α6 transfected cells compared to si-Scr transfected cells whereas the cell surface expression of β1 was unchanged in cells transfected with either si-α6 or si-Scr oligonucleotides. Isotype-control immunoprecipitations performed for each antibody were negative (data not shown). These data indicate that siRNA oligonucleotides for α6 effectively reduce the level of α6β4 expression on the cell surface of breast carcinoma cells. In addition, these findings substantiate the fact that most, if not all, β4 expressed on the cell surface is associated with α6.

Given our finding that si-α6 oligonucleotides efficiently decrease the level of α6β4 on the cell surface of MDA-MB-231 cells, we investigated the ability of si-α6 oligonucleotides to inhibit their invasion and migration. The invasion of si-α6 transfected cells through Matrigel towards LPA was decreased by 53% compared to cells transfected with si-Scr oligonucleotides (Figure 4B). In additional experiments, we found a similar reduction in invasion when fibroblast conditioned medium was used as the chemoattractant (data

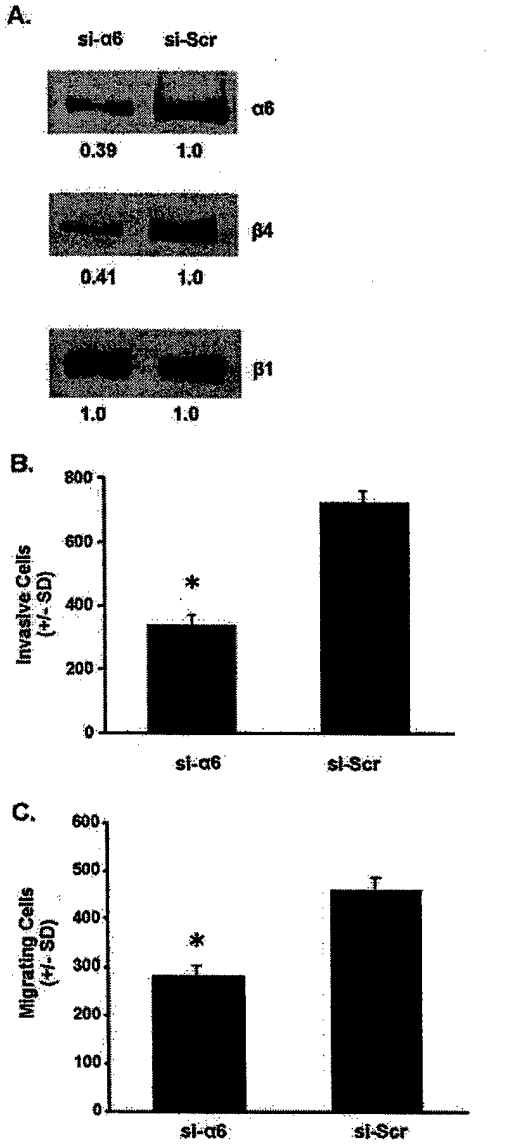

Figure 4. si-α6 oligonucleotides decrease invasion and migration of MDA-MB-231 cells. (A) Three days following transfection with si-α6 or si-Scr oligonucleotides, MDA-MB-231 cells were biotinylated, extracted in RIPA buffer, and immunoprecipitated for α6, β4, and β1. Each immunoprecipitation was initiated with 125 μg of total protein and separated by 6% SDS-PAGE. The membranes were incubated with peroxidase-conjugated streptavadin. The fold change in integrin expression following si-α6 transfection is shown in comparison to the si-Scr transfected band. (B) The ability of si-α6 and si-Scr transfected MDA-MB-231 cells to invade Matrigel towards LPA in a 4 h assay was assessed. The results represent the mean number of invasive cells (+/− SD) from two wells (five fields per well). The mean number of invasive cells was significantly decreased for si-α6 transfected cells compared to si-Scr transfected cells (*, two-tailed *t*-test, $p = 0.0002$). Similar results were obtained in three independent trials. (C) At 72 h post-transfection with si-α6 or si-Scr oligonucleotides, the migration of these cells through collagen I-coated Transwell membranes towards LPA (100 nM) for 1 h was determined. The data represent the mean number of migrating cells (+/− SD) from two wells (five fields per well). The number of migrating cells was significantly less for si-α6 transfected cells than for si-Scr transfected cells (* two-tailed *t*-test, $p = 0.001$). Similar results were obtained in two separate experiments.

not shown). The level of apoptosis was comparable for both si-α6 and si-Scr transfected cells as determined by the percentage of AnnexinV-PE$^+$ cells (data not shown). To assess the ability of si-α6 and si-Scr transfected cells to migrate in a ligand-independent manner, we performed migration assays towards LPA on collagen I-coated Transwells. The mean number of migrating cells was decreased by 39% in the si-α6 transfected cells compared to the si-Scr transfected cells (Figure 4C). These results provide more definitive evidence for the ability of $\alpha6\beta4$ to stimulate migration on non-laminin substrata. Overall, these data emphasize the importance of $\alpha6\beta4$ in the invasive phenotype of breast carcinoma cells.

Discussion

The relatively new technique of RNAi is a potentially powerful tool to assess the contribution of specific molecules to invasion and metastasis. In particular, the specificity and efficacy of this approach may be especially valuable for studying the integrin family members that exhibit complex structures and multiple functions. To assess the feasibility of RNAi in this capacity, we focused on the $\alpha6\beta4$ integrin. The involvement of this integrin in invasion and migration has been previously established by expression of the $\beta4$ subunit in $\beta4$-deficient cell lines and by function blocking antibodies. In this study, we provide the first evidence that the integrins can be inhibited by the more definite approach of RNAi and suggest that targeting of the $\alpha6\beta4$ integrin may be an effective strategy to assess the functions of this integrin and impede carcinoma progression.

The utilization of siRNA oligonucleotides targeted to the $\alpha6$ and $\beta4$ subunits of the $\alpha6\beta4$ integrin provided valuable information not only for the usefulness of RNAi to inhibit surface receptors but also on the mechanistic function of this integrin. We found that the endogenous expression of $\alpha6\beta4$ was not decreased until 72 or 96 hours post-transfection with si-α6 and si-$\beta4$ oligonucleotides, respectively. This observation indicates that surface receptors may require considerable time for the RNAi approach to be effective and suggests that the half-life of the target protein and the rate of surface receptor recycling are major determinants in the ability of RNAi to inhibit expression. In contrast, our laboratory has shown that soluble proteins are inhibited efficiently by RNAi, usually within 24–48 hours after transfection (R.E. Bachelder, unpublished observation). In functional assays, reduced $\alpha6\beta4$ expression resulted in significant decreases in invasion and migration even though the inhibition of $\alpha6\beta4$ expression was less than complete. If the expression of $\alpha6\beta4$ were further diminished, the effects would likely be more dramatic. Because the reduction in $\alpha6\beta4$ expression in these studies is dependent on both transfection efficiency and the specific gene sequence selected for RNAi inhibition, manipulation of either of these parameters may ultimately lead to greater reduction in $\alpha6\beta4$ expression. For example, the use of different siRNA oligonucleotides targeted to either of the $\alpha6\beta4$ subunits may provide more complete inhibition. In addition, the recently developed DNA vector-based RNAi technology [27–29] which allows for stable inhibition of the targeted gene will eliminate transfection efficiency concerns and should also permit the generation of $\alpha6\beta4$ deficient cell lines. The implementation of these strategies will likely enable us to achieve greater reduction in $\alpha6\beta4$ expression in future studies.

Data from our laboratory and others have recently implicated the $\beta4$-subunit in ligand-independent signaling. For example, the $\alpha6\beta4$ integrin promotes migration and invasion, as well as lamellae formation, on non-laminin substrata such as collagen and these functions cannot be blocked by antibodies that inhibit $\alpha6\beta4$ adhesive interactions, an observation that discounts the possibility of adhesion to laminins deposited by the cells [10]. More recently, it was demonstrated that a truncated form of $\beta4$ that was unable to bind laminins could promote invasion [11]. In this study, the migration of MDA-MB-231 cells on collagen I, a non-ligand for $\alpha6\beta4$, was significantly inhibited by si-$\beta4$ and si-α6 oligonucleotides thus providing further evidence that this integrin can function in the absence of ligand. This concept has profound implications for migration and invasion because it implies that the ability of $\alpha6\beta4$ to stimulate these pathways is not limited to specific matrix environments. Although the mechanism by which $\alpha6\beta4$ functions in this manner is unknown, the observation that $\beta4$ cytoplasmic domains can self-associate may explain this ligand-independent signaling [30]. Furthermore, $\alpha6\beta4$ has been shown to regulate the function of $\alpha3\beta1$, a dual collagen/laminin receptor, in keratinocytes [31, 32]. Even though we did not observe a change in either $\alpha3$ or $\beta1$ subunit expression in our studies, we cannot rule out the possibility that $\alpha6\beta4$ affects $\alpha3\beta1$-mediated signaling to cause decreased migration on collagen I in si-$\beta4$ and si-α6 transfected cells.

Because $\alpha6\beta4$ is an adhesion receptor for laminin-1 as well as other laminins [9], we hypothesized that loss of this integrin would promote a decrease in adhesion to this substrata. However, we observed an increase in adhesion to laminin-1 following transfection with si-$\beta4$ oligonucleotides. This seeming paradox is explained by our finding that the α6 subunit forms additional $\alpha6\beta1$ complexes in the absence of the $\beta4$ subunit. This finding is further supported by the observation that the $\alpha6\beta4$ integrin is decreased with either si-$\beta4$ or si-α6 oligonucleotides but that cells transfected with si-α6 oligonucleotides have reduced levels of the $\beta4$ subunit whereas the si-$\beta4$ oligonucleotides do not significantly decrease the expression of the α6 subunit. Thus, the α6 subunit is stabilized on the cell surface by associating with $\beta1$ subunits that are found expressed in excess in many cells but the $\beta4$ subunit, that associates exclusively with the α6 subunit [12], is lost from the cell surface when the α6 subunit is removed.

Several groups have recently demonstrated that siRNA oligonucleotides can inhibit gene expression *in vivo*. For example, mouse tail vein injections with siRNA oligonucleotides for luciferase, delivered as naked siRNA oligonucleotides or as plasmid DNA, in combination with a luciferase expression vector significantly reduced luciferase expression in adult mice [33]. In similar studies, the endogenous expression of the green fluorescent protein was

decreased in the liver of a transgenic mouse strain that expresses this gene in nearly all organs [34]. Furthermore, the therapeutic potential of this technique to inhibit cancer progression was specifically shown by the lack of tumor formation in nude mice that were injected with cells that had been selected for stable and reduced expression of oncogenic K-RAS [35]. Taken together, these studies indicate that RNAi may be a viable approach to treat human diseases including cancer.

In summary, we have shown that siRNA oligonucleotides can reduce the endogenous surface expression of the $\alpha 6\beta 4$ integrin in carcinoma cells. In the absence of $\alpha 6\beta 4$, we observed a significant reduction in the invasion and ligand-independent migration of these cells. These results demonstrate the validity of this technique to inhibit integrin expression in carcinoma cells and suggest that the $\alpha 6\beta 4$ integrin may be a potential gene target to prevent tumor progression *in vivo*. Future studies by our laboratory will directly explore this possibility in mouse cancer models.

Acknowledgements

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JCB Article

Integrin ($\alpha6\beta4$) regulation of eIF-4E activity and VEGF translation: a survival mechanism for carcinoma cells

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We define a novel mechanism by which integrins regulate growth factor expression and the survival of carcinoma cells. Specifically, we demonstrate that the $\alpha6\beta4$ integrin enhances vascular endothelial growth factor (VEGF) translation in breast carcinoma cells. The mechanism involves the ability of this integrin to stimulate the phosphorylation and inactivation of 4E-binding protein (4E-BP1), a translational repressor that inhibits the function of eukaryotic translation initiation factor 4E (eIF-4E). The regulation of 4E-BP1 phosphorylation by $\alpha6\beta4$ derives from the ability of this integrin to activate the PI-3K–Akt pathway and, consequently, the rapamycin-sensitive kinase mTOR that can phosphorylate 4E-BP1. Importantly, we show that this $\alpha6\beta4$-dependent regulation of VEGF translation plays an important role in the survival of metastatic breast carcinoma cells by sustaining a VEGF autocrine signaling pathway that involves activation of PI-3K and Akt. These findings reveal that integrin-mediated activation of PI-3K–Akt is amplified by integrin-stimulated VEGF expression and they provide a mechanism that substantiates the reported role of $\alpha6\beta4$ in carcinoma progression.

Introduction

An understanding of the mechanisms that sustain the survival of tumor cells in adverse physiological conditions is one of the most important problems in cancer biology. As argued recently, cancer progression is an evolutionary process that selects for cells that exhibit the capacity for survival in environmental conditions not present in normal tissue (Fearon, 1999; Hanahan and Weinberg, 2000). One survival strategy used by tumor cells is the secretion of proteins that elicit an angiogenic response, such as vascular permeability factor or vascular endothelial growth factor (VEGF).* VEGF appears to be an essential factor for the progression of many solid tumors (Shweiki et al., 1992; Brown et al., 1999; Dvorak et al., 1999). It is widely assumed that the function of VEGF produced by tumor and tumor stromal cells is to stimulate angiogenesis by acting in a paracrine fashion on vicinal endothelium (Hanahan and Folkman, 1996; Brown et al., 1999). Another mechanism for tumor cell survival is the establishment of autocrine signaling loops that act on tumor cells directly (Scotlandi et al., 1996; Tokunou et al., 2001; Wong et al., 2001). Although the significance of this mechanism has been overshadowed by angiogenesis, recent studies have substantiated the importance and necessity of such signaling loops for tumor survival (Scotlandi et al., 1996; Bachelder et al., 2001; Tokunou et al., 2001; Wong et al., 2001). Indeed, this mechanism probably contributes to the ability of cells to survive in hypoxic, poorly vascularized regions of tumors. In this direction, we described recently the existence of a VEGF autocrine signaling pathway in metastatic breast carcinoma cells that is essential for their survival (Bachelder et al., 2001).

An important issue that arises from the contribution of VEGF autocrine signaling to tumor survival is an understanding of the mechanisms that regulate VEGF expression. Such mechanisms are important not only for VEGF signaling in tumor cells, but also for angiogenesis as well. Clearly, hypoxia is a strong inducer of VEGF transcription and mRNA stability (von Marschall et al., 2001), but other factors are likely to be involved. Of note, our finding that the $\alpha6\beta4$ integrin can promote the survival of breast carcinoma cells in stress conditions is intriguing (Bachelder et al., 1999b) and raised the novel possibility that a specific integrin, which has been implicated in cancer progression, could regulate VEGF expression. This possibility is substantiated by the finding reported here that the ability of the $\alpha6\beta4$ integrin to promote survival is VEGF dependent.

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*Abbreviations used in this paper: 4E-BP1, 4E-binding protein; eIF-4E, eukaryotic initiation factor-4E; mTOR, mammalian target of rapamycin; Myr-Akt, myristoylated Akt; PI, propidium iodide; PI-3K, phosphatidylinositol 3-kinase; RNAi, small interfering RNA; VEGF, vascular endothelial growth factor.

Key words: integrin; VEGF; translation; carcinoma; eIF-4E

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The results described above prompted us to investigate the relationship between the α6β4 integrin and VEGF expression. We observed that the expression and signaling properties of this integrin have no impact on steady-state VEGF mRNA levels. Surprisingly, however, we detected a significant influence of α6β4 on VEGF translation and protein expression in these cells, an observation that reveals the ability of this integrin to regulate translation. The mechanism by which α6β4 regulates VEGF expression involves its ability to stimulate the phosphorylation of 4E-binding protein (4E-BP1). 4E-BP1 is phosphorylated by mammalian target of rapamycin (mTOR), a protein kinase whose catalytic domain is structurally related to that of phosphatidylinositol 3-kinase (PI-3K) (Dennis et al., 1999; Schmelzle and Hall, 2000). Phosphorylation of 4E-BP1 by mTOR disrupts its binding to eukaryotic translation initiation factor eIF-4E, which is present in rate-limiting amounts in most cells (De Benedetti and Harris, 1999; McKendrick et al., 1999). eIF-4E plays a critical role in the recruitment of the translational machinery to the 5′ end of mRNA, which is demarcated by an m7GpppN cap (where m is a methyl group and N is any nucleotide) (Raught and Gingras, 1999). The m7 cap is essential for the translation of most mRNAs including VEGF (De Benedetti and Harris, 1999; Raught and Gingras, 1999). Dissociation of 4E-BP1 from eIF-4E enables eIF-4E to initiate translation (Gingras et al., 1999, 2001b). The regulation of 4E-BP1 phosphorylation by α6β4 derives from the ability of this integrin to activate the PI-3K–Akt pathway and, consequently, mTOR. Our findings reveal a novel mechanism of tumor cell survival and they highlight the ability of a specific integrin to regulate protein translation by influencing eIF-4E activity.

Results

The ability of the α6β4 integrin to promote the survival of carcinoma cells is VEGF dependent

To examine the hypothesis that the ability of the α6β4 integrin to promote survival is VEGF dependent, we used MDA-MB-435 cells, which lack expression of this integrin. Stable expression of α6β4 in these cells enhances their ability to survive in stressful conditions (Bachelder et al., 1999b). Importantly, however, expression of α6β4 does not alter the expression of other integrin subunits in these cells and does not influence their adhesion to matrix (Shaw et al., 1997). As shown in Fig.1 A, a significant level of apoptosis was observed after 24 h of serum deprivation in the parental MDA-MB-435 cells and mock transfectants, as well as in transfectants that express α6β4 containing a cytoplasmic domain deletion of the β4 subunit that lacks the ability to signal (Shaw et al., 1997). Stable subclones that express the intact α6β4 integrin, however, were protected from apoptosis under these conditions. Based on these results and our previous finding that the survival of metastatic breast carcinoma cells is dependent on VEGF, we used a VEGF antisense oligonucleotide to reduce VEGF expression in the MDA-MB-435/β4 transfectants and assessed the impact of reducing VEGF expression on their survival (Fig. 1, B and C). The VEGF antisense oligonucleotide reduced VEGF protein expression significantly in the β4 transfectants (Fig. 1 C). As shown in Fig. 1 B, this reduction in VEGF expression abrogated the survival-enhancing effect of α6β4 under conditions of serum deprivation.

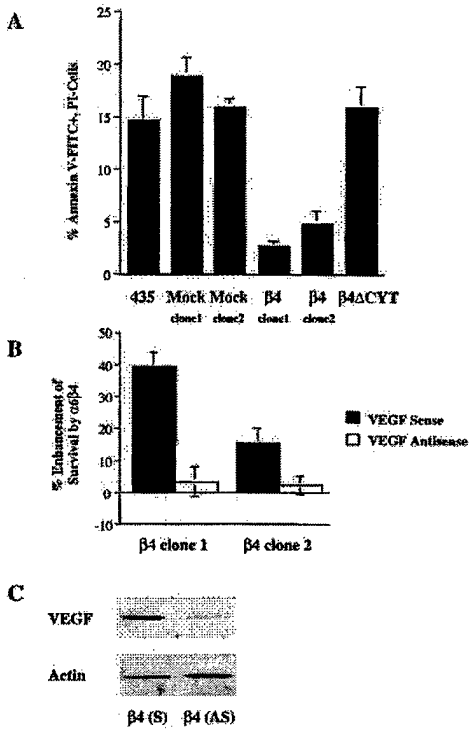

Figure 1. **The α6β4-mediated survival of breast carcinoma cells is VEGF dependent.** (A) Parental, mock (clone 1, 6D2; clone 2, 6D7), β4-ΔCYT–expressing (cytoplasmic tail deletion mutant), and β4 integrin–expressing (clone 1, 3A7; clone 2, 5B3) MDA-MB-435 subclones were maintained in low serum (0.5% FBS) medium for 24 h. To assess the level of apoptosis, these cells were stained with annexin V–FITC and propidium iodide (PI), and analyzed on a Becton Dickinson flow cytometer using CellQuest software. The percentage of annexin-positive, PI-negative cells (± SD) is indicated. Results were obtained from three independent experiments. Apoptosis was minimal in the presence of 10% FBS (unpublished data). (B) Mock-transfected clone 6D7 and β4 integrin– (clone 1, 3A7; clone 2, 5B3) expressing MDA-MB-435 subclones were transiently transfected with VEGF sense or antisense oligonucleotides and maintained in low serum (0.5% FBS) medium. After 24 h, the level of apoptosis in these cells was assessed as described above. The data are presented as the mean difference (± SD) in annexin positivity between mock-transfected and α6β4-expressing MDA-MB-435 cells. Similar results were observed in two separate experiments. (C) The relative amount of VEGF protein in extracts obtained from the MDA-MB-435/β4 cells transfected with either the VEGF sense (S) or antisense (AS) oligonucleotide was determined by immunoblotting using a polyclonal anti-VEGF immune serum.

The α6β4 integrin increases VEGF protein but not mRNA expression

Given that the survival effect of α6β4 expression is VEGF dependent, the novel possibility arose that VEGF expression could be regulated by this integrin. VEGF expression can be regulated at the level of both transcription and mRNA stability (Nabors et al., 2001; von Marschall et al., 2001), mechanisms that would alter the steady-state levels of VEGF

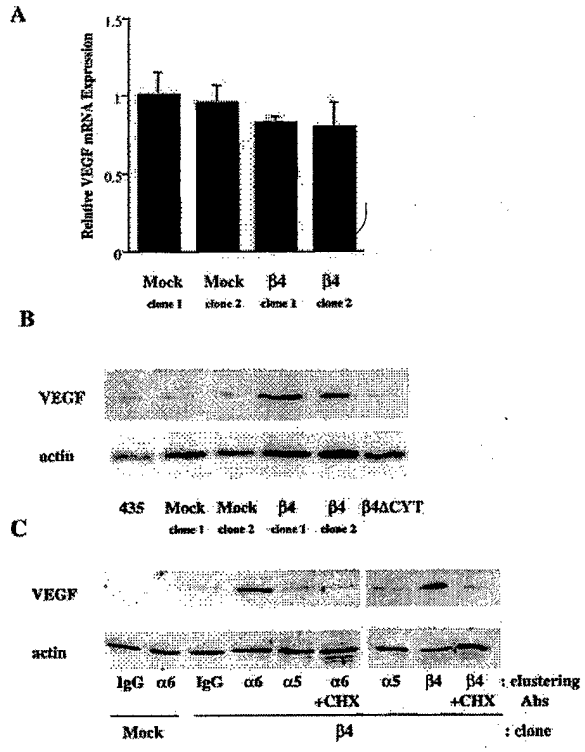

Figure 2. **Expression of the α6β4 integrin increases VEGF protein but not steady-state mRNA.** (A) The amount of VEGF mRNA in extracts obtained from mock- (clone 1, 6D2; clone 2, 6D7) and β4 integrin– (clone 1, 3A7; clone 2, 5B3) transfected MDA-MB-435 subclones was quantified by real-time PCR. The data are presented as the mean ratio of VEGF to β-actin mRNA (± SD) obtained from triplicate samples. (B) Parental (435), mock (clone 1, 6D2; clone 2, 6D7), β4-ΔCYT–expressing (clone 1E10), and β4 integrin–expressing (clone 1: 3A7, clone 2: 5B3) MDA-MB-435 subclones were cultured in low serum (0.5% FBS) medium for 24 h. Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. Similar results were observed in four independent experiments. (C) Mock (clone 6D7) and β4 integrin–expressing (clone 3A7) MDA-MB-435 subclones were maintained in low serum (0.5% FBS) medium for 24 h. These cells were detached with trypsin and incubated with integrin-specific antibodies (α6 integrin, 2B7; β4 integrin, A9; α5 integrin, Sam1) or IgG for 30 min in suspension and allowed to adhere on anti-IgG–coated plates for 60 min before lysis. In addition, cells were preincubated in cycloheximide (CHX) at a concentration of 10 μg/ml for 30 min and then incubated with either the α6 or β4 integrin antibodies in the presence of cycloheximide. Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. Similar results were observed in two independent experiments.

mRNA. In addition, regulation can also occur at the level of VEGF translation (Kevil et al., 1996; Akiri et al., 1998; Stein et al., 1998). As shown in Fig. 2 A, quantitative analysis of VEGF mRNA levels in two clones of MDA-MB-435/mock and β4 transfectants using real-time PCR revealed no significant difference in the steady-state mRNA levels in these two populations. However, we detected a substantial increase in VEGF protein expression in the MDA-MB-435/β4 transfectants relative to either the parental cells, mock transfectants, or cells that express a cytoplasmic domain deletion of the β4 subunit (β4-ΔCYT) (Fig. 2 B). These results indicate that the α6β4 integrin regulates VEGF protein expression. It is also worth noting that the level of apoptosis observed in these populations in response to serum deprivation correlates inversely with their expression of VEGF (Fig. 1 A and Fig. 2 B).

To substantiate the regulation of VEGF expression by α6β4, integrin-specific antibodies were used to cluster either α6β4 or α5β1 and the effects of integrin-mediated clustering on VEGF expression were assessed by immunoblotting. Of note, the MDA-MB-435/β4 transfectants express equivalent levels of α6β4 and α5β1 (unpublished data). An α6-specific antibody (mAb 2B7) was used to cluster the α6β1 integrin in the mock transfectants and the α6β4 integrin in the β4 transfectants, a β4-specific antibody (mAb A9) was used to cluster the α6β4 integrin in the β4 transfectants, and an α5-specific antibody (mAb Sam1) was used to cluster α5β1 in both the mock and β4 transfectants. A substantial induction of VEGF expression was observed upon α6β4 integrin clustering in the β4 transfectants but not in the mock transfectants, and no induction was seen in response to α5β1 clustering (Fig. 2 C). Importantly, the induction of VEGF expression that occurs in response to α6β4 clustering was inhibited by cycloheximide (Fig. 2 C). This result, together with the real-time PCR data (Fig. 2 A), indicates that α6β4 is influencing VEGF translation.

To obtain more definitive evidence that α6β4 is regulating VEGF translation, we performed polysome analysis of the VEGF message. mRNA isolated from the MDA-MB-435/mock and β4 transfectants was fractionated on a sucrose gradient (Fig. 3 A) and the relative amount of VEGF mRNA in each fraction was determined by real-time PCR (Fig. 3 B). As shown in Fig. 3 B, a striking difference in the distribution of VEGF mRNA was evident in the two populations of cells. In the MDA-MB-435/β4 transfectants, VEGF mRNA fractionated in the heavy polysomal region, whereas in the mock transfectants, the majority of VEGF mRNA was associated with light polysomal to ribosomal subunit fractions. This result indicates that the translation of VEGF in the MDA-MB-435/β4 transfectants is cap dependent.

Identification of an α6β4 integrin–mediated signaling pathway that regulates VEGF expression

Our finding that α6β4 regulates the cap-dependent translation of VEGF prompted us to assess the ability of this integrin to stimulate the activity of the eIF-4E translation initiation factor. The α6β4 integrin is a potent activator of the PI-3K–Akt signaling pathway in MDA-MB-435 and other carcinoma cells (Shaw et al., 1997; Bachelder et al., 1999a; Gambaletta et al., 2000; Nguyen et al., 2000; Hintermann et al., 2001), and this pathway has been linked to the regulation of protein translation. Specifically, the serine/threonine kinase mTOR is activated by Akt-mediated phosphorylation events (Sekulic et al., 2000). Phosphorylation of 4E-BP1 by mTOR disrupts its binding to eIF-4E, enabling eIF-4E to initiate translation of VEGF and other molecules (De Benedetti and Harris, 1999). We hypothesized, based on this information, that α6β4 regulates 4E-BP1 phosphorylation and, as a consequence, VEGF expression. Initially, we as-

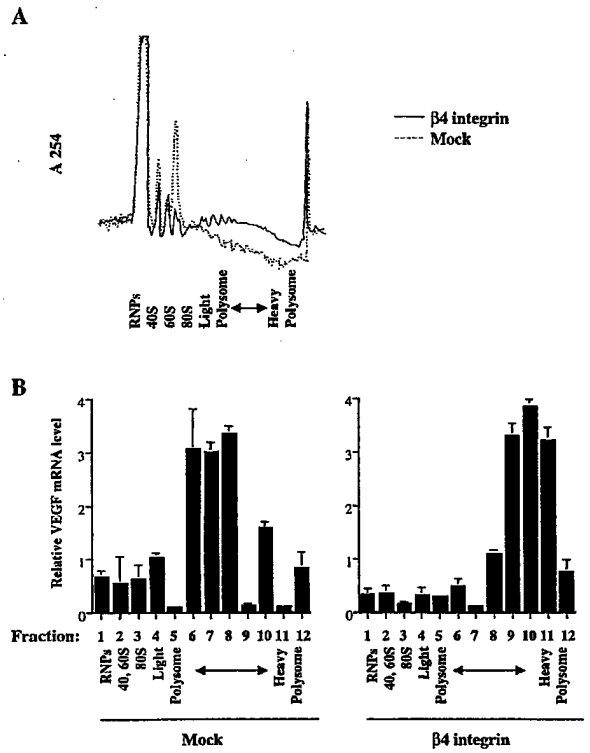

Figure 3. **Polysome analysis of VEGF mRNA.** (A) The distribution of RNA from MDA-MB-435/β4 and mock transfectants that had been fractionated on sucrose gradients as described in the Materials and methods was determined by measuring the A_{254} of each fraction. (B) The relative VEGF mRNA content of each sucrose gradient fraction was measured by real-time PCR as described in the Materials and methods. Fraction 1 contains unbound RNA present in the ribonucleoprotein fraction, fraction 2 contains 40S and 60S monosomes, fraction 3 contains 80S monosomes, fractions 4–7 contain light polysomes, and fractions 8–12 contain heavy polysomes. The data are presented as the mean ratio of VEGF to β-actin mRNA (± SD) obtained from triplicate samples. Similar results were obtained from three independent experiments.

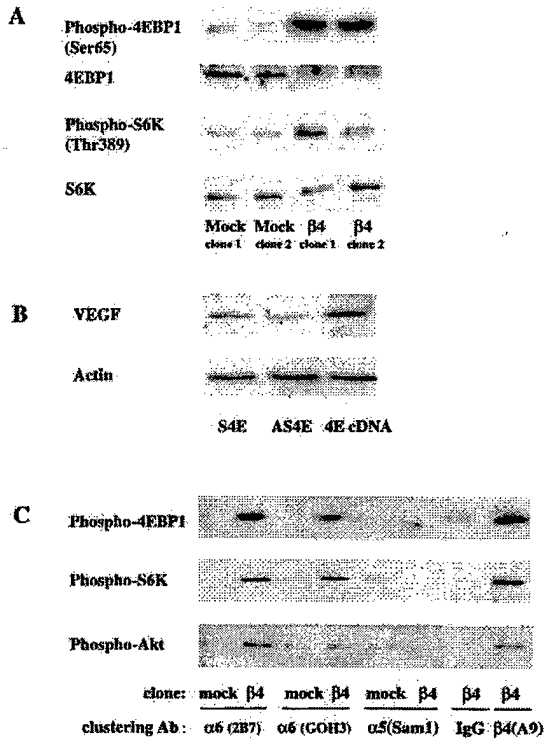

Figure 4. **The α6β4 integrin stimulates the phosphorylation of Akt, 4E-BP1, and p70^{S6K}.** (A) MDA-MB-435 parental cells, mock transfectants, and β4 transfectants were maintained in medium containing low serum (0.5% FBS) for 24 h. The phosphorylation status of 4E-BP1 on Ser 65 and S6K on Thr 389 was assessed in extracts from these cells using phosphospecific antibodies as described in the Materials and methods. In addition, the total amount of 4E-BP1 and p70^{S6K} in these extracts was assessed by immunoblotting. (B) The MDA-MB-435/β4 cells were transiently transfected with either an eIF-4E sense (S) or antisense (AS) oligonucleotide, or a full-length eIF-4E cDNA (4E). Extracts of these cells containing equivalent amounts of protein were analyzed for their relative expression of VEGF and actin by immunoblotting. (C) MDA-MB-435 mock (clone 6D7) and β4 (clone 3A7) transfectants were maintained in low serum (0.5% FBS) medium for 24 h. These cells were detached with trypsin and incubated with integrin-specific antibodies (α6 integrin, 2B7; α6 integrin, GOH3; α5 integrin, Sam1; β4 integrin, A9) or IgG for 30 min as described in the legend to Fig. 2. The phosphorylation status of 4E-BP1 (Ser 65), S6K (Thr 389), and Akt (Ser 473) was assessed in extracts from these cells using phosphospecific antibodies. Similar results were observed in four independent experiments.

sessed the steady-state phosphorylation levels of 4E-BP1 and S6 kinase (p70^{S6K}), which are both downstream targets of mTOR, in cells that had been serum deprived for 24 h. Indeed, a marked increase in the level of phosphorylation of 4E-BP1 (on Ser65) and p70^{S6K} (on Thr389) was evident in the MDA-MB-435/β4 transfectants relative to either the mock transfectants or the parental cells (Fig. 4 A). Phosphorylation of Ser65 of 4E-BP1 has been shown to be critical for dissociation of 4E-BP from eIF-4E (Gingras et al., 2001a). The reduced expression of 4E-BP1 in the β4 transfectants compared with the mock transfectants that is apparent in Fig. 4 A may reflect the possibility that the 4E-BP Ab does not recognize the hyperphosphorylated form of the protein as well as it recognizes the hypophosphorylated form.

The involvement of eIF-4E in VEGF translation was confirmed by the expression of an antisense eIF-4E oligonucleotide in the MDA-MB-435/β4 transfectants. As shown in Fig. 4 B, expression of this oligonucleotide reduced the level of VEGF protein significantly. In contrast, expression of the full-length eIF-4E cDNA increased the VEGF protein level by approximately twofold. These results, together with the polysome analysis data (Fig. 3), indicate that α6β4 regulates VEGF expression by eIF-4E–mediated, cap-dependent translation.

To confirm the specificity of the α6β4 integrin in mTOR signaling, the effects of integrin-mediated clustering on 4E-BP1 phosphorylation were assessed. A substantial induction of Akt, 4E-BP1, and p70^{S6K} phosphorylation was observed upon α6β4 integrin clustering in the β4 transfectants but not in the mock transfectants (Fig. 4 C). In contrast, clustering of the α5β1 integrin did not stimulate phosphorylation of these molecules in either the mock or β4 transfectants. Collectively, these data demonstrate the preferential ability of the α6β4 integrin to regulate the mTOR signaling pathway and, more importantly, the phosphorylation of 4E-BP1.

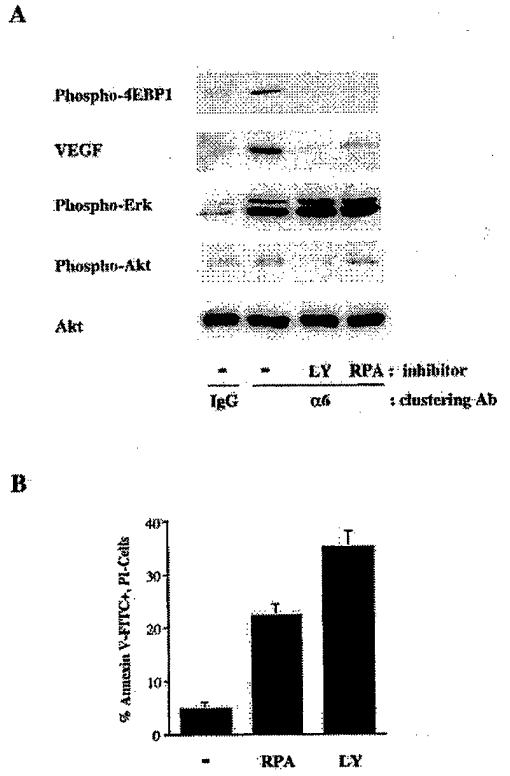

Figure 5. **Stimulation of 4E-BP1 phosphorylation, VEGF expression, and survival by the α6β4 integrin requires PI-3K and mTOR.** (A) MDA-MB-435 β4 transfectants (clone 3A7) were incubated with either DMSO (−), the PI-3K inhibitor LY 294002 (10 μM) (LY), or the mTOR-specific inhibitor rapamycin (50nM) (RPA) for 30 min and then incubated with either IgG or the α6 integrin antibody 2B7 as described in the legend to Fig. 2. Extracts of these cells were immunoblotted for phospho–4E-BP1 (Ser65), VEGF, phospho-Erk (recognizing phosphorylated isoforms of ERK1 and ERK2), phospho-Akt (Ser 473), and total Akt. Similar data were obtained in three experiments. (B) MDA-MB-435 β4 transfectants (clone 3A7) were maintained at low serum (0.5%) medium for 24 h in the presence of either rapamycin (50nM) (RPA), LY 294002 (10 μM) (LY), or DMSO (−). Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of a representative experiment performed in triplicate.

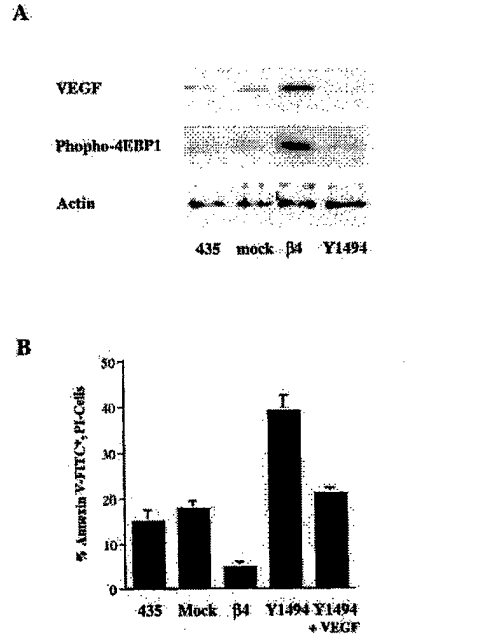

Figure 6. **Y1494 in the β4 cytoplasmic domain is required for α6β4 stimulation of 4E-BP1 phosphorylation, VEGF expression, and survival.** (A) MDA-MB-435 parental cells (435), mock transfectants (clone 6D7), wild-type β4 transfectants (clone 3A7), and Y1494F mutant transfectants (clone E1h) were maintained in low serum (0.5% FBS) for 24 h. Extracts from these cells were analyzed by immunoblotting to assess the relative expression of VEGF and 4E-BP1 phosphorylation. The relative amount of actin was also determined as a control for protein loading. Similar results were obtained in three experiments. (B) Aliquots of the same cell populations described in A were assayed for the level of apoptosis after a 24-h incubation in low serum (0.5% FBS) medium. Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of three experiments performed in triplicate.

To establish that PI-3K and mTOR are required for 4E-BP1 phosphorylation and VEGF expression, we performed the antibody clustering experiments in the presence of the PI-3K–specific inhibitor LY294002 and the mTOR-specific inhibitor rapamycin (Fig. 5). As shown in Fig. 5 A, both of these inhibitors blocked the α6β4-mediated induction of 4E-BP1 phosphorylation and VEGF expression. Although rapamycin did not block Akt phosphorylation, LY294002 did inhibit its phosphorylation, confirming that Akt acts upstream of mTOR and downstream of PI-3K (Fig. 5 A). These inhibitors did not block the phosphorylation of ERK1 and ERK2 (Fig. 5 A).

Finally, we investigated the importance of the mTOR pathway in survival, using rapamycin and LY294002. As shown in Fig. 5 B, rapamycin treatment increased the apoptosis of the MDA-MB-435/β4 transfectants fivefold and LY294002 treatment increased their apoptosis eightfold. These results indicate that the PI-3K–mTOR pathway is critical for the survival of these cells.

Identification of a specific tyrosine residue in the β4 cytoplasmic domain required for α6β4 stimulation of 4E-BP1 phosphorylation and VEGF expression

Recently, a critical tyrosine residue (Y1494) was identified in the third fibronectin type III repeat of the β4 cytoplasmic domain, and this tyrosine was shown to be essential for activation of PI-3K by α6β4 (Shaw, 2001). To assess the importance of Y1494 in 4E-BP1 phosphorylation and VEGF expression, stable subclones of MDA-MB-435 cells were generated that expressed α6β4 containing a Y1494F mutation. As shown in Fig. 6 A, VEGF protein expression was barely detectable in these transfectants compared with the wild-type transfectants. Also, the steady-state level of 4E-BP1 phosphorylation was substantially lower in the Y1494F mutant transfectants than in the wild-type β4 transfectants. Interestingly, these mutant transfectants also exhibited an

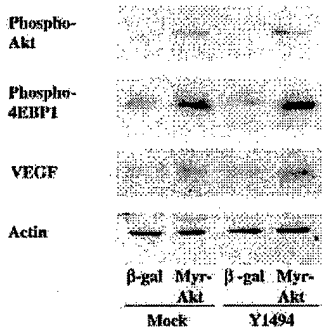

Figure 7. **Expression of a constitutively active Akt construct mimics the effects of α6β4 integrin expression and signaling.** MDA-MB-435 mock transfectants (clone 6D7) and Y1494F mutant transfectants (clone E1h) were infected with adenoviruses that expressed either β-galactosidase or Myr-Akt as described in the Materials and methods. Subsequently, the cells were incubated in low serum (0.5% FBS) medium for 24 h. Extracts of these cells were immunoblotted to assess the relative phosphorylation of Akt and 4E-BP1, as well as total expression of VEGF and actin.

eightfold higher level of apoptosis than the wild-type β4 transfectants in response to serum deprivation (Fig. 6 B). The apoptosis of the mutant cells was reduced substantially by the addition of recombinant VEGF (Fig. 6 B), a result that substantiates the importance of VEGF in the survival of these cells. Together, these findings highlight the importance of the β4 cytoplasmic domain and PI-3K signaling in the regulation of VEGF expression and tumor cell survival.

Expression of constitutively active Akt stimulates 4E-BP1 phosphorylation and VEGF expression in the absence of α6β4 signaling

The hypothesis that activation of Akt is a major determinant for the stimulation of 4E-BP1 phosphorylation and VEGF expression was assessed by expressing a constitutively active Akt construct in MDA-MB-435 cells that are deficient in α6β4 signaling. For this purpose, we used MDA-MB-435/mock transfectants that lack α6β4 expression and the MDA-MB-435/β4 Y1494F transfectants, described above, which are deficient in α6β4-mediated activation of PI-3K. These cells were infected with adenoviruses that encoded either a myristoylated Akt (Myr-Akt) construct or β-galactosidase as a control. As shown in Fig. 7, expression of Myr-Akt stimulated 4E-BP1 phosphorylation and VEGF expression substantially in both populations of transfectants in comparison to cells that expressed β-galactosidase. This result indicates the critical importance of Akt activation by α6β4 for stimulating VEGF expression.

α6β4 regulates 4E-BP1 phosphorylation, VEGF expression, and survival in carcinoma cells that express this integrin endogenously

Given that the data reported above are based on the exogenous expression of α6β4 in α6β4-deficient carcinoma cells, we sought to extend our findings to cells that express this integrin endogenously, a pattern that is typical of most carcinoma cells. For this purpose, we used MDA-MB-231 breast carcinoma cells because they express the α6β4 and α5β1 integrins (Plopper et al., 1998; Mukhopadhyay et al., 1999; Saad et al., 2000). Initially, we confirmed that the survival of these cells is dependent on their expression of VEGF. As shown in Fig. 8 A, expression of a VEGF antisense oligonucleotide in these cells (Bachelder et al., 2001) resulted in an approximate fourfold increase in annexin V staining upon serum starvation compared with either untreated cells or cells that expressed the sense oligonucleotide.

To establish a role for α6β4 in regulating VEGF expression and survival rigorously, we used a small interfering

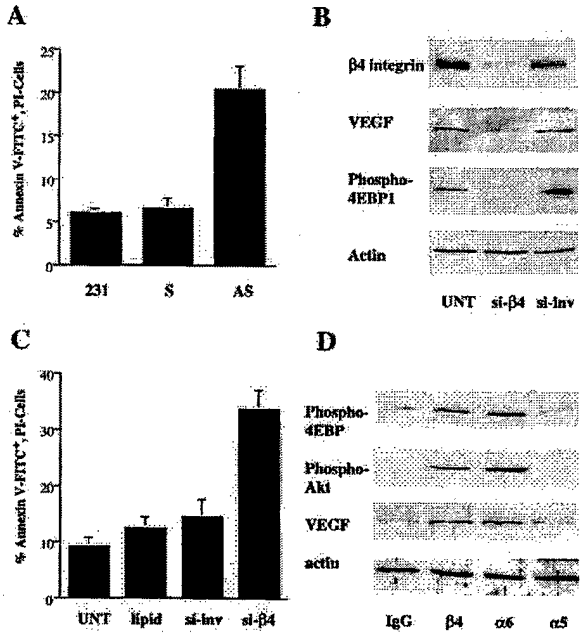

Figure 8. **α6β4 regulates 4E-BP1 phosphorylation, VEGF expression, and survival in carcinoma cells that express this integrin endogenously.** (A) Parental MDA-MB-231 cells and cells transfected with antisense or sense VEGF oligonucleotides were maintained in low serum (0.5% FBS) medium for 24 h. Apoptosis was assayed as described in the Materials and methods and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of two separate experiments performed in triplicate. (B) MDA-MB-231 cells were left untreated (UNT) or were transfected with either an RNAi specific for the β4 integrin (si-β4) or the corresponding inverted sequence (si-inv). After 72 h, the cells were placed in medium containing low serum (0.5% FBS) for an additional 24 h and then extracted. Extracts of these cells were immunoblotted as described in the legend to Fig. 4 to assess expression of β4 integrin, VEGF, and actin, as well as the phosphorylation of 4E-BP1. Similar results were observed in three independent trials. (C) Apoptosis was assessed in the same populations of cells and is reported as the percentage of annexin V–FITC-positive, PI-negative cells. The data shown are mean values (± SD) of three independent experiments performed in triplicate. (D) MDA-MB-231 cells were maintained in low serum (0.5% FBS) medium for 24 h and harvested by trypsin treatment. The suspended cells were incubated with integrin-specific antibodies (β4 integrin, A9; α6 integrin, 2B7; α5 integrin, Sam1) or IgG for 30 min in suspension and allowed to adhere on anti-IgG–coated plates for 30 min. Extracts of these cells were immunoblotted with phosphospecific antibodies to assess the relative phosphorylation of Akt and 4E-BP1, as well as with antibodies specific for VEGF and actin. Similar results were obtained in five experiments.

RNA (RNAi) approach to inhibit β4 expression in MDA-231 cells. RNAis specific for the β4 subunit and the corresponding inverted sequence were designed and expressed in these cells by transfection. The cells were maintained in low serum (0.5%) for 24 h after transfection and then analyzed. As shown in Fig. 8 B, cells transfected with the RNAi specific for β4 exhibited a significant reduction in β4 expression in comparison with either untransfected cells or cells transfected with the inverted sequence. Importantly, the reduction in β4 expression by RNAi coincided with a marked reduction in 4E-BP1 phosphorylation and in the steady-state level of VEGF (Fig. 8 B), as well as an approximate threefold increase in annexin V staining (Fig. 8 C). These results link α6β4 expression directly to 4E-BP1 phosphorylation, VEGF expression, and survival in a carcinoma cell line that expresses endogenous α6β4.

Subsequently, we performed antibody clustering experiments to substantiate the regulation of VEGF expression by α6β4 (Fig. 8 D). Clustering of the α6β4 integrin with either an α6 integrin–specific antibody (mAb 2B7) or a β4 integrin–specific antibody (mAb A9) stimulated the phosphorylation of 4E-BP1 and Akt, and increased VEGF expression. In contrast, no induction of VEGF expression or stimulation of either 4E-BP1 or Akt phosphorylation was observed upon clustering with an α5 integrin–specific antibody (mAb Sam1) or IgG.

Discussion

This study establishes a novel mechanism by which integrins regulate growth factor expression. Specifically, our findings demonstrate the ability of a specific integrin (α6β4), which has been implicated in carcinoma progression (Mercurio and Rabinovitz, 2001), to stimulate the translation of VEGF and sustain a VEGF autocrine loop that is essential for survival. More specifically, we define a signaling pathway regulated by α6β4 that involves the preferential ability of this integrin to stimulate the phosphorylation of 4E-BP1 by activating the PI-3K–Akt pathway. As shown previously, this phosphorylation event dissociates 4E-BP1 from eIF-4E, enabling this key elongation factor to mediate the translation of VEGF and other functionally important molecules (De Benedetti and Harris, 1999; Gingras et al., 1999, 2001b; McKendrick et al., 1999). Moreover, the polysome analysis and antisense eIF-4E results we provide indicate that α6β4 stimulation of VEGF translation is cap dependent and probably doesn't involve the internal ribosome entry sites that are present in the VEGF mRNA (Huez et al., 1998; van der Velden and Thomas, 1999). Our data extend earlier reports on the involvement of eIF-4E, VEGF, and α6β4 in carcinoma progression by linking these molecules in a common signaling pathway that promotes tumor survival. Furthermore, they reveal a role for integrins in regulating growth factor expression by stimulating protein translation.

An important and novel aspect of our findings is that they add a new dimension to the understanding of how integrins promote cell survival. The widely accepted notion is that integrins, often in concert with growth factor receptors, activate specific signaling pathways that sustain survival (Taylor et al., 1999; Liu et al., 2000). We demonstrate here that the survival function of integrins may not only be mediated by the activation of a key survival kinase such as Akt and the consequent effects of Akt on apoptotic signaling (Datta et al., 1999) but also by the Akt-dependent translation and expression of growth factors, such as VEGF, that promote survival in an autocrine, and possibly paracrine, fashion. In other terms, our results reveal that VEGF is a novel target of Akt signaling by integrins that is important for survival and distinct from known survival factors that are downstream of Akt, such as Bad (Datta et al., 1999). Importantly, our recent observation that VEGF stimulates the PI-3K–Akt pathway in carcinoma cells (Bachelder et al., 2001), in conjunction with our finding that α6β4 signaling enhances VEGF expression, leads to the conclusion that integrin-mediated activation of PI-3K–Akt is amplified by integrin-stimulated VEGF expression. Moreover, we show that this amplification of PI-3K–Akt activity is important for carcinoma survival.

Although α6β4 activates PI-3K in carcinoma cells (Gambaletta et al., 2000; Nguyen et al., 2000; Hintermann et al., 2001; Trusolino et al., 2001), no attempt had been made to link this signaling event with downstream effectors that regulate protein translation, namely mTOR and 4E-BP1. One reason that this possibility had not been explored is because a role for α6β4 in regulating either protein translation or growth factor expression was not obvious. In fact, almost all of the functional studies on α6β4 in carcinoma cells have focused on its role in promoting migration and invasion, and on the mechanism by which α6β4-mediated signaling influences these processes (Mercurio, 1990; Shaw et al., 1997; Gambaletta et al., 2000; Trusolino et al., 2001). Our motivation to examine a possible connection between α6β4 and VEGF translation was based on our interest in understanding the mechanisms by which these molecules promote the survival of carcinoma cells. Indeed, our results establish a role for α6β4 in survival signaling by regulating VEGF translation, but the implications of these findings are more widespread. For example, recent studies that have argued that α6β4 is necessary for growth factor receptor (erbB2, c-met) activation of PI-3K (Gambaletta et al., 2000; Trusolino et al., 2001) raise the interesting possibility of an intimate functional association among specific growth factor receptors, α6β4, VEGF, and PI-3K, all of which have been implicated in tumor progression.

Surprisingly, few studies have examined the role of integrin signaling in regulating protein translation (e.g., Pabla et al., 1999). Indeed, there has been much more interest in defining the contribution of integrins to transcription. The ability of integrins to regulate translation, however, provides a mechanism for altering cell function rapidly, by increasing the expression of specific proteins. This possibility is exemplified by our finding that ligation of the α6β4 integrin resulted in a significant increase in VEGF protein within 60 min (Fig. 2 C). Given the fact that eIF-4E is rate limiting for the translation of proteins involved in growth control and other critical cell functions (De Benedetti and Harris, 1999), the hypothesis can be formulated that integrin-mediated regulation of translation contributes to the ability of cells to alter their behavior rapidly in response to changes in their microenvironment. This hypothesis is particularly relevant to our interest in the regulation of VEGF expression. Al-

though much of the work in this area has focused on the ability of hypoxia to stimulate VEGF transcription and increase the stability of VEGF mRNA (von Marschall et al., 2001), it has become apparent that translational control is also important (Kevil et al., 1996; De Benedetti and Harris, 1999). Moreover, our recent finding that VEGF is essential for the survival of breast carcinoma cells in normoxia substantiates the functional importance of integrin-mediated regulation of VEGF expression (Bachelder et al., 2001).

The fact that our data implicate eIF-4E in tumor cell survival is of considerable interest because recent studies have revealed an important role for this elongation factor in cancer (DeFatta et al., 1999, 2000; Ernst-Stecken, 2000; Berkel et al., 2001). Overexpression of this factor in NIH3T3 cells, as well as other "normal" cells, stimulates division and can induce their transformation (Fukuchi-Shimogori et al., 1997). These findings are consistent with the reports that the expression of eIF-4E is elevated in solid tumors compared with normal tissue (De Benedetti and Harris, 1999). Moreover, hypoxia, a pathophysiological stress that provides a selective pressure for the survival of aggressive tumor cells, enhances eIF-4E expression (DeFatta et al., 1999). Together, these observations highlight an important role for translational control in human cancer. This role is substantiated by the fact that eIF-4E controls the translation of not only VEGF but also other molecules that influence tumor growth and survival such as c-Myc, cyclin D1, and FGF-2 (De Benedetti and Harris, 1999). From our perspective, we are intrigued by the reports that the α6β4 integrin is associated with the progression of many solid tumors, and its expression has been correlated with a poorer prognosis in patients with some of these tumors (Mercurio and Rabinovitz, 2001). Our finding that α6β4 can induce the translational function of eIF-4E by regulating the phosphorylation of 4E-BP1 provides one mechanism to account for the role of this integrin in cancer.

Materials and methods

Cells

MDA-MB-231 and MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University. They were grown in low glucose DME containing 10% FBS, 1% penicillin–streptomycin, and 25 mM Hepes. For inhibitor experiments, cells were harvested by trypsinization and suspended cells were incubated with rapamycin (Calbiochem) at 100 nM or LY 294002 (Calbiochem) at 10 μM on ice for 30 min before they were plated at 37°C for the experiment.

The generation of MDA-MB-435 subclones expressing the α6β4 integrin has been described previously (Shaw et al., 1997). Tyrosine residue 1494 in the β4 subunit was mutated to a phenylalanine residue using the Quickchange site-directed mutagenesis kit (Stratagene), and stable subclones of MDA-MB-435 cells that expressed α6β4 containing this mutant β4 subunit were generated (Shaw, 2001).

For adenoviral infection, cells were grown in DME containing 10% FBS until they reached 50% confluency. At this point, the culture medium was changed to DME containing 0.5% FBS. Viral dilutions were prepared from purified viral stocks in DME containing 0.5% FBS and the cells were infected for 4 h. At the end of the infection period, the virus-containing medium was removed and the cells were washed once with PBS, and incubated for an additional 12 h in DME containing 10% FBS.

Apoptosis assays

To induce apoptosis, cells were incubated in DME containing 0.5% FBS for 24 h at 37°C. Subsequently, both adherent and nonadherent cells were harvested and their level of apoptosis was assessed using annexin V–FITC. In brief, cells were washed once with serum-containing medium, once with PBS, once with annexin V–FITC buffer (10 mM Hepes-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and then incubated for 15 min at room temperature with 5 μg/ml annexin V–FITC (Biosource International). After washing once with annexin V buffer, the samples were resuspended in the same buffer and analyzed by flow cytometry. Immediately before the analysis, 5 μg/ml propidium iodide (PI) (Biosource International) was added to distinguish apoptotic cells from necrotic cells.

Quantitative real-time PCR

Quantitative analysis of VEGF mRNA expression was performed by real-time PCR using an ABI Prism 7700 sequence detection system (Perkin-Elmer) and SYBR green master mix kit as described previously (Bachelder et al., 2001). Sequences of primers and probes were as follows: VEGF forward primer, 5′-GAAGTGGTGAAGTTCATGGATGTCTA-3′; VEGF reverse primer, 5′-TGGAAGATGTCCACCAGGGT-3′; VEGF probe, 5′-/TET/AGCGCAGCTACTGCCATCCAATCG/TAM/-3′; β-actin forward primer, 5′-TCACCATGGATGATGATATCGC-3′; β-actin reverse primer, 5′-AAGCCGGCCTTGCACAT-3′; and β-actin probe, 5′-/FAM/CGCTCGTCGTCGACAACGGCT/TAM/-3′.The data obtained are presented as the mean ratio of VEGF to β-actin mRNA (± SD) obtained from triplicate samples.

VEGF antisense oligonucleotide experiments

A VEGF antisense 2′-*O*-methyl phosphorothioate oligodeoxynucleotide (5′-CACCCAAGACAGCAGAA-3′) and a sense 2′-*O*-methyl phosphorothioate oligodeoxynucleotide (5′-CTTTCTGCTGTCTTGGGTG) (provided by Greg Robinson, Children's Hospital, Boston, MA) were used to transfect MDA-MB-435 β4 transfectants at a concentration of 0.3 μM in the presence of lipofectin reagent (2 μg/ml; GIBCO BRL). The experimental details for this approach have been described previously (Bachelder et al., 2001). In addition, the same protocol was used to express antisense and sense eIF-4E oligonucleotides, which were gifts from Arigo De Benedetti (Louisiana State University, Shreveport, LA) (DeFatta et al., 2000).

RNAi experiments

An RNAi specific for the β4 integrin subunit (GAGCUGCACGGAGUGUGUC) as well as the inverted sequence (CUGUGUGAGGCACGUCGAG) were designed and synthesized by Dharmacon, Inc. MDA-231 cells at 30% confluency were transfected with 300 pmoles of RNAi using TKO lipids (Mirus). Subsequently, the cells were maintained in complete medium for 72 h and in medium containing 0.5% FBS for an additional 24 h before analysis.

Polysome analysis

Cells (3×10^7) were maintained in medium containing low serum (0.5% FBS) for 24 h and then pretreated with 100 μg/ml cycloheximide (Calbiochem) for 15 min at 37°C before being harvested. After washing once with PBS containing 100 μg/ml cycloheximide, the cells were resuspended in 0.5 ml of a modified U+S buffer (Davies and Abe, 1995). This buffer was composed of 200 mM Tris-HCl (pH 8.8), 25 mM $MgCl_2$, 5 mM EGTA (pH 8.0), 150 mM KCl, 10 μg/ml heparin, 5 mM DTT, 1% sodium deoxycholate, 2% polyoxyethylene 10-tridecy ester, 100 μg/ml cycloheximide, and 200 mM sucrose. Ribonuclease inhibitor (Amersham Biosciences) was added to a final concentration of 0.5 U/μl. Cells were homogenized with 20–25 strokes in a Kontes tissue homogenizer, followed by centrifugation for 5 min at 14,000 *g*. The supernatant was collected and frozen at −80°C until further use. Sucrose gradients (15–50%, wt/wt) were layered with 300 μl of cleared cell extract, which was then centrifuged at 160,000 *g* for 2 h. Fractions (0.75–0.375 ml) were withdrawn from the top of the gradient and monitored for absorbency at 254 nm using an ISCO syringe pump with UV-6 detector. Total RNA from the sucrose gradient fractions was extracted using Trizol LS (Life Technologies) according to the manufacturer's instructions. Quantitative real-time PCR was used to measure the VEGF mRNA level in each fraction as described above.

Integrin signaling experiments

Cells were harvested by trypsin treatment and washed twice with DME containing 25 mM Hepes and 0.1% BSA. After washing, the cells were resuspended in the same buffer at a concentration of 2×10^6 cells/ml and incubated for 30 min with integrin-specific antibodies (4 μg/ml) or with either mouse or rat IgG (4 μg/ml). The cells were washed once, resuspended in the same buffer, and added to plates that had been coated overnight with either the anti–mouse or rat IgG. After a 60-min incubation at 37°C, the cells that had attached to integrin-specific antibodies were washed twice with cold PBS and solubilized at 4°C for 10 min using RIPA buffer (20 mM Tris buffer, pH 7.4, containing 0.14 M NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, 5 μg/ml aprotinin,

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pepstatin, and leupeptin). The IgG-treated cells were harvested by centrifugation and solubilized with RIPA buffer.

Protein analysis

Aliquots of cell extracts containing equivalent amounts of protein were solubilized using 5× sample buffer containing 100 mM DTT and then incubated at 100°C for 15 min. These extracts were resolved by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked for 1 h using a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 0.05% Tween-20 (TBST), and 5% (wt/vol) Carnation dry milk. The filters were incubated overnight in the same buffer containing antibodies specific for p70S6K, 4EBP antibodies (Santa Cruz Biotechnology, Inc.), actin (ICN Biomedicals), and VEGF (clone 618, provided by Donald Senger, Beth Israel Deaconess Medical Center). After three, 10-min washes in TBST, the filters were incubated for 1 h in blocking buffer containing HRP-conjugated secondary antibodies. After three 10-min washes in TBST, proteins were detected by ECL (Pierce Chemical Co.).

For immunoblots involving phosphospecific antibodies, the filters were blocked for 1 h using a 10 mM Tris buffer, pH 7.5, containing 0.5 M NaCl, 0.1% Tween-20, and 2% (wt/vol) BSA. The filters were washed briefly and then incubated overnight at 4°C in the same blocking buffer containing antibodies specific for phospho-p70S6K (Thr-389; Cell Signaling Technology), phospho–4E-BP1 (Ser-65; Cell Signaling Technology), phospho-Erk (E10; Cell Signaling Technology), and phospho-Akt (Ser-473 clone 4E2; Cell Signaling Technology). After washing, the filters were incubated for 1 h in blocking buffer containing HRP-conjugated secondary antibody and the proteins were detected by ECL.

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